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SUMARIO

A Study of the Afferent Path of the Hypoglycemic Reflex of Insulin. — R. Álvarez Buylla, E. T. Segura and Elena R. de Álvarez Buylla 43

Hematic Values in Residents at Altitudes Between 1260 m and 4515 m Above Sea Level. — H. Chiodi and C. Pozzi 51

La Fibrilación Ventricular en el Corazón Perfundido del Perro. — E. López y A. Gaxiola 60

Administration of Pellets of Morphine to Mice; Abstinence Syndrome. — C. Maggiolo and F. Huidobro 70

Vascular Changes in the Retina of Diabetic Rats. — I. T. L. Musacchio, N. Palermo and R. R. Rodríguez 79

La Acomodación del Músculo Ventricular de Tortuga. — G. Pilar y A. Gaxiola 84

The Variation of the Electric Resistance of Microelectrodes During the Flow of Current. — R. Rubio and G. Zubieta 91

Metabolism of Malto-Oligosaccharides. — H. N. Torres and J. M. Olavarría 95

Proceedings of the Society of Biology of Ribeirão Prêto, São Paulo, Brazil 106

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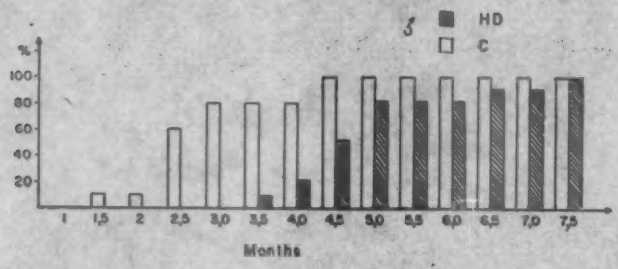

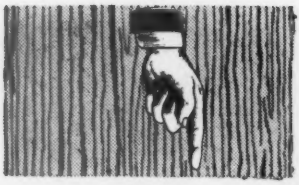

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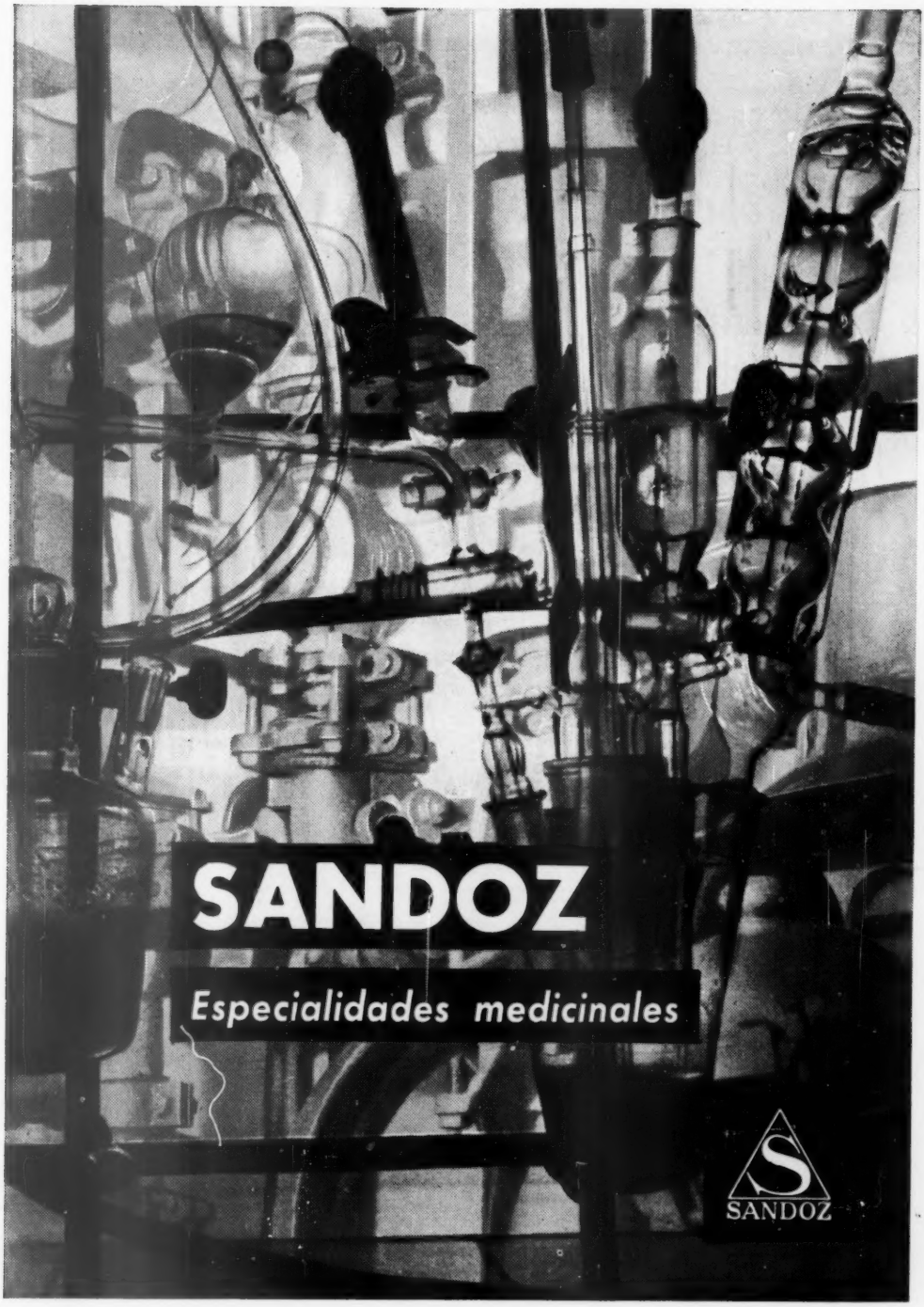
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C. H. BEST y N. B. TAYLOR

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A STUDY OF THE AFFERENT PATH OF THE HYPOGLYCEMIC REFLEX OF INSULIN

R. Álvarez-Buylla, E. T. Segura (*) and
Elena R. de Álvarez-Buylla
(National Institute of Neumology of México, Department of Physiology, México)

By injecting insulin intravenously a given number of times coincident with the sound of a bell, a conditioned reflex is established by which the sensorial stimulus is converted to an afferent signal initiating a hypoglycemic reflex; this hypoglycemic reflex is similar in magnitude to that provoked by the insulin dosage used in the reinforcements (Savchenko, 1939; Leites and Pavlov, 1954; Álvarez-Buylla et al., 1959; Álvarez-Buylla and Carrasco Zanini, 1960).

On the basis of these experiments, it was assumed that the hypoglycemic effect of insulin might include in its own mechanism a nervous component, in addition to the known humoral effect of insulin.

Further studies established that conditioned hypoglycemia is not due to an increased production of endogenous insulin, since the reflex can also be established in the totally pancreatectomized dog (Álvarez-Buylla and Carrasco Zanini, 1960). In the spinal dog, with the spine sectioned at C 6 and C 7, this hypoglycemic reflex was observed to be similar to that produced in the intact animals (Álvarez-Buylla, E., 1959).

The present work was undertaken to study the role of the vagus as a possible afferent path of this conditioned reflex.

Materials and methods

Dogs, cats and rabbits were used.

The training of the spinal dogs was started two weeks after the spinal cord was sectioned at C 6 and C 7, utilizing for each reinforcement 0.25 units/kg of insulin in aqueous solution given intravenously. Once the conditioned hypoglycemic reflex had been established (with at least two positive controls), vagotomy was performed through an abdominal incision at the diaphragm level. One week after vagotomy, the animals had recovered sufficiently for further studies in the chamber for conditioned reflex.

For the experiments on the perfusion of the splanchnic area, the donor cat was eviscerated, the proximal ends of

(*) Fellow of the "Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina".

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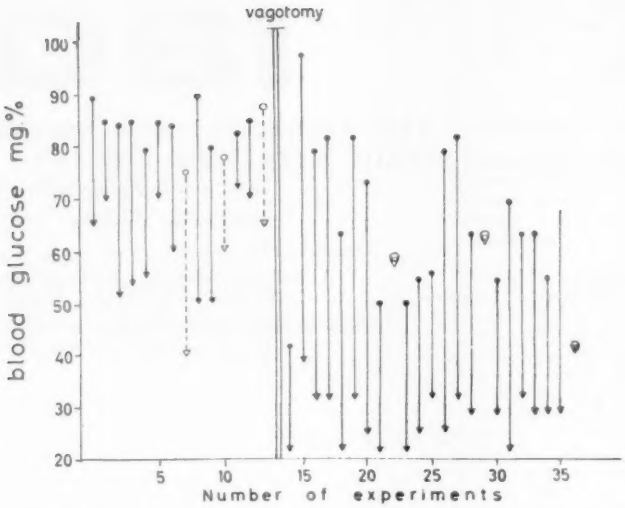

FIG. 1. — *Conditioned hypoglycemic reflex in the spinal dog and its disappearance following abdominal vagotomy.* Circles: *control samples of basal glycemia.* Triangles: *glycemia 15 minutes after control samples were drawn.* Solid circles and solid lines: *experiments using intravenous injection of insulin (0.25 U/kg) associated with bell's sound.* Open circles and dotted lines: *control experiments using distilled water and bell's sound.*

the inferior vena cava and of the abdominal aorta were catheterized immediately below the diaphragm of the donor cat and joined to the distal ends of the inferior vena cava and the abdominal aorta of the receptor cat. The proximal ends of these vessels were also ligated and sectioned directly below the diaphragm. The cats were heparinized systemically, and the adrenal vessels were ligated. As anesthetic, pentobarbital (40 mg/kg) was given intraperitoneally.

One hour later blood samples were drawn from the carotids of both animals, and insulin was immediately injected through the tube which joined the two aortas. The amount varied between 0.02 and 0.5 units/kg. Samples were taken simultaneously from the carotids of both animals 15 and 30 minutes after injection.

In the five dogs having indwelling polyethylene catheters inserted within a branch of the celiac trunk, insulin (0.5 units/kg) tolerance curves were determined. In all cases the curves were determined at least 24 hours after the catheter had been inserted, in isolated, unanesthetized animals as close as possible to basal conditions.

Finally, rabbits and dogs were anesthetized with pentobarbital (45 mg/kg in the rabbits and 30 mg/kg in the dogs) to study the effects on glycemia of insulin injected 1) in the splanchnic area, 2) following complete abdominal vagotomy, and 3) after electrical stimulation of the proximal ends of the vagus. To determine the A. V. glucose difference, thin polyethylene catheters were placed in the femoral artery and vein, from which blood samples were taken. For the injection of insulin, a

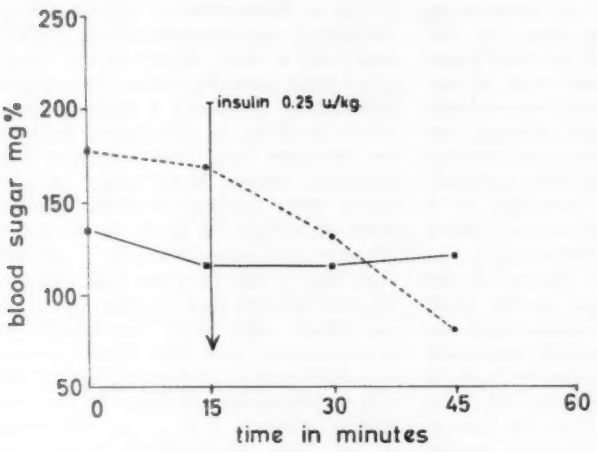

FIG. 2. — *Average curve of experiments showing insulin effect when injected in the splanchnic area of a receptor cat perfused by the donor cat.* Solid line: *donor cat.* Dotted line: *receptor cat.*

similar catheter was introduced into the splenic artery until the end of the catheter was approximately 1 cm from the celiac trunk. Control samples were taken simultaneously from the femoral vein and artery after a period of one hour and again 15 min later. Three series of experiments were then conducted to determine 1) changes in the A. V. glucose difference secondary to the injection of insulin in the splenic artery (0.0005 to 0.5 units/kg); 2) changes in the A. V. glucose difference secondary to the injection of insulin following abdominal vagotomy; 3) the effects of electrical stimulation of the proximal ends of some of the vagal branches in the abdomen. These proximal ends were mounted on bi-polar platinum electrodes and stimulated for 30 seconds at a frequency of 20/second with rectangular impulses of 2 milliseconds duration and an intensity approximately three times the threshold.

The Nelson-Somogyi method was used for all blood glucose determinations. The samples were taken at 2, 4, 8 and 16 minutes following stimulation, induced by injection of insulin, vagal section, or through electrical stimulation of the proximal vagal stump.

RESULTS

A. Effects of vagotomy on the conditioned hypoglycemic reflex in the spinal dog.

Since the conditioned hypoglycemic reflex in the spinal dog (with spinal chord transection at C 6 and C 7) is similar to that established in the normal dog, the spinal chord is eliminated as a possible afferent path of this reflex.

Figure 1 shows that the conditioned hypoglycemia established in the spinal animal by pairing a bell sound with the injection of insulin (0.25 units/kg) for seven trials, disappeared after abdominal vagotomy, even though the insulin dosage was quadrupled (1 unit/kg).

B. Injection of insulin in the perfused splanchnic area.

Granted that the mechanism of the action of insulin has a reflex com-

ponent, the possibility of dissociating the nervous component from the humoral one was studied in seven experiments. The splanchnic area of one cat (the receptor animal) was isolated from the rest of the body although the nervous connections were maintained intact; the circulation of the splanchnic area of this cat was provided by a second animal (the perfusor cat) which had been previously eviscerated.

Figure 2 shows the effects of the aortic injection of insulin on the blood glucose level of the non-perfused superior half. The abdominal aorta was used to perfuse the inferior half of such an animal. Blood samples were drawn simultaneously from the carotid arteries of both animals for glucose determinations.

As shown, while the arterial blood glucose level of the donor cats (solid line) showed no significant changes, the concentration of glucose in the carotid artery of the receptor cats (dotted line) decreased 22.1 % in 15 minutes following the injection of insulin, falling to 52 % after 30 minutes. Four additional experiments were performed with a much lower dosage of insulin (0.02 units/kg). After 15 minutes this dosage produced a hypoglycemic effect of 23 % in the upper half of the receptor cats. Two receptor cats with an average blood sugar of 110 mg % were used as controls. Insulin (0.03 units/kg) was given in the jugular vein, this dosage being 50 % greater than that of the previous experiments. Fifteen minutes post insulin injection, the blood sugar level determined on blood drawn from the carotid artery was 108.5 mg %, indicating that there was no hypoglycemic effect in the two control animals. The 1.5 %, difference is less than the method's margin of error.

These results indicate that the marked hypoglycemia of the head and trunk of the receptor cat as a consequence of insulin injected in the circulatorily isolated splanchnic area was not related to the small amount of insulin that might reach the lead-trunk

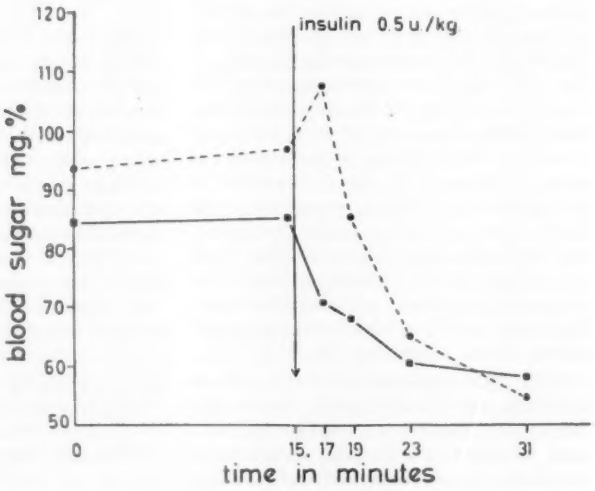

FIG. 3. — *Unanesthetized dogs.* Dotted line: *arterial glycemia.* Solid line: *venous glycemia. Arrow indicates the injection of insulin 0.5 U/kg in one of the celiac artery branches. Each point is the average of 10 experiments. Standard deviations were small (or non significant).*

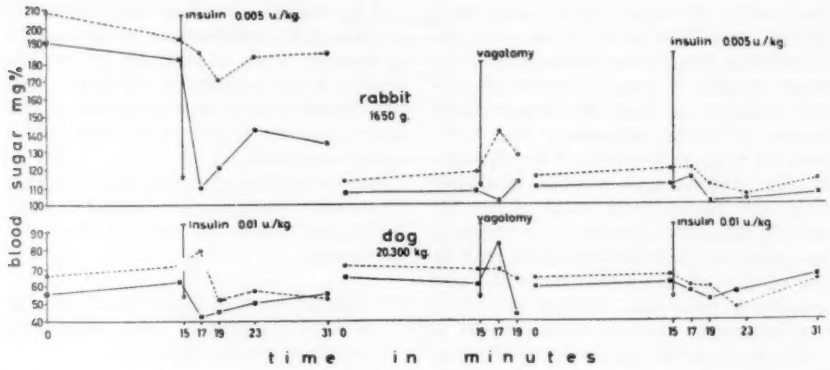

FIG. 4. — *Effects of insulin injections in the celiac trunk before and after vagotomy.*

circulation. Higher doses of insulin injected directly in that area produced no hypoglycemic reflex in a 15 minute period. Since this hypoglycemia must start along a nervous path, the nervous supply of the splanchnic area was maintained intact in these experiments.

C. Effects of the injection of insulin in the arterial celiac trunk in unanesthetized dogs.

To obtain additional data on the participation of the vagus in the reflex action of insulin, insulin was injected in the celiac trunk so that the hormone would reach, via the celiac artery, the assumed insulin-sensitive receptors.

In 5 unanesthetized dogs with permanent catheters in the celiac trunk, 20 insulin tolerance curves were made. The glucose concentration in the catheterized artery was determined in 10 curves; in the remaining 10, glucose was determined on the specimen of venous blood from the external saphenous vein.

Arterial and venous samples were drawn 2, 4, 8, and 16 minutes following the injection of insulin (0.5 units/kg). Figure 3 shows the average curves obtained. Each dot represents the arithmetic average of the 10 cases. The glucose content of the arterial blood showed sudden changes during the first minutes after the injection of insulin with an initial peak followed by a significant hypoglycemia. The venous blood showed an immediate and significant hypoglycemia, without the initial hyperglycemic peak seen in the arterial samples. These results demonstrate that the injection of insulin in the celiac trunk mobilizes the glucose to the muscles in two minutes; it is probably then stored as muscular glycogen since the posterior extremities received arterial blood with a glucose concentration slightly higher than before the injection (96 mg % to 108 mg %). In the venous blood it is lower than before the injection of insulin (87 mg % to 70 mg %). The posterior extremity was used in these experiments because of its high proportion of muscular tissue.

D. Effects of the injection of insulin in the arterial celiac trunk in anesthetized dogs and rabbits.

To determine whether insulin produces in anesthetized animals the hypoglycemic reflex observed in unanesthe-

tized dogs, 10 experiments were conducted on rabbits and 11 on dogs. In the rabbits, insulin injected in the celiac trunk produced quantitatively significant changes in both the arterial and venous glycemia, increasing the A. V. difference in every case. The changes in the arterial and venous glycemia were very rapid since in 17 of the 20 insulin tests, the changes were marked two minutes after the injection and in two of the 20, after 4 minutes. In the remaining case, this change appeared 8 minutes after the injection of insulin.

In the dogs the results were similar. The arterial glycemia changed according to the patterns already seen in 81 % of the cases (9 of 11 experiments) and the venous glycemia showed similar changes, but in 80 % (8 out of 10) of the cases. In 7 instances the changes were apparent in 2 minutes and in one at 4 minutes.

In 19 of the 20 experiments (9 on rabbits, 11 on dogs), or in 95 % of the cases, the increased A. V. difference was apparent in the first two minutes following the injection of insulin. These results indicate that the anesthesia did not eliminate the hypoglycemic reflex initiated by insulin.

E. Abdominal vagotomy and the injection of insulin in the arterial celiac trunk.

The effects of the complete section of the vagus near its entrance to the abdomen were studied in 11 experiments, 7 on dogs, 4 on rabbits. As a result of the section, the arterial glycemia changed rapidly and significantly in 9 of the 11 cases, the venous in 10 of 10, and the A. V. difference in 9 of 10. No constant variation was observed although the most common was the dissociation of the arterial hyperglycemia and the venous hypoglycemia curves. There were cases in which the glycemia increased or decreased in the same direction in both vessels.

The effects of abdominal vagotomy on the reflex mechanism of the action of insulin were studied in 10 experiments, 4 on rabbits, 6 on dogs. The injections of insulin were given in the same manner before and after abdominal vagotomy.

Following vagotomy the reflex action of insulin disappeared. Figure 4 illustrates the results of two of these experiments.

F. Effects of the stimulation of the central stump of the abdominal vagus nerve on glycemia.

Twenty-two experiments were carried out, 6 on rabbits, 16 on dogs. The branches of the vagal trunk to the right of the esophagus at the level of the diaphragm were used because they gave more uniform and constant responses to stimuli, than those to the left.

In all 22 cases, the vagal stimulation lasting 30 seconds or a minute produced in the rabbits as well as in the dogs, a sharp increase in the A. V. difference in the concentration of glucose. In almost all cases, this change was very rapid. Figure 5 shows the results of two of these experiments.

Discussion

The work by Houssay and co-workers indicates that the homeostasis of glucose is not controlled exclusively by the pancreas but that many other endocrine components are involved, mainly the hypophysis. Bernard, in 1857, had already made observations on the influence of the nervous system, and specifically of the vagus nerve, on glycemia.

The problem of the hypothalamus-hypophysis relationship has not yet been solved (see the review by Harris) [5]. The fact that the hypoglycemic effect of insulin can be reproduced by a stimulus, such as a bell sound, chosen

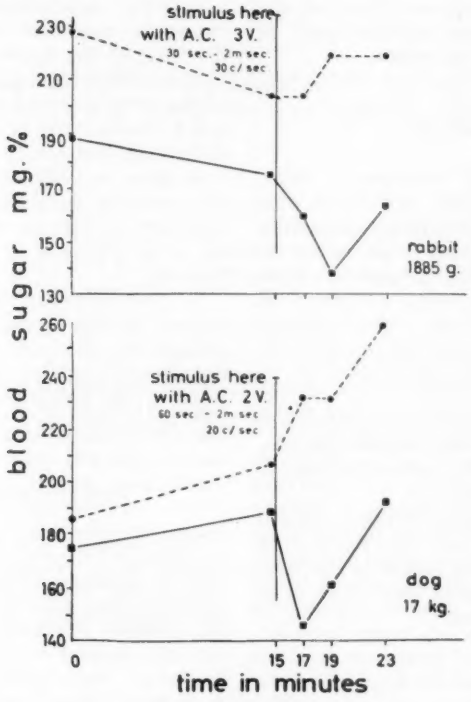

FIG. 5. — *Effect of the stimulation of vagus' proximal and on the A-V glucose difference in dogs and rabbits.* Dotted line: *arterial glycemia.* Solid line: *venous glycemia.*

haphazardly opens a new and valuable experimental approach to the problem of the neuro-humoral integration and its probably important rôle in glucose homeostasis.

When taken together the results of the various experiments described in this paper, the participation of the vagus in this reflex is confirmed. As a matter of fact, the injection of insulin in the perfused splanchnic area, with the vagal nerves intact, initiates a hypoglycemic reflex in the head and trunk, whose circulation remains almost independent (see Figure 2). Insulin injected in the celiac trunk of unanesthetized animals produces marked effects on glycemia in less than two minutes (Figure 3). These effects disappear following vagotomy, a fact which also shows vagal participation in this reflex action of insulin (Figure 4). To analyze this vagal participation, the proximal ends of the abdominal vagus which has been previously sectioned were electrically stimulated (Figure 5), and an alteration in the glycemic reflex occured with an increase in the A. V. difference with a latency of less than a minute. All these facts lead to the conclusion that

some of the abdominal fibers of the vagal nerve are the afferent path of this insulin-triggered reflex.

Summary

1) After several intravenous injections (0.25 units/kg) coincident with a bell, a conditioned reflex is established; the sound alone without insulin produces a definite hypoglycemia similar to the hypoglycemia produced by the insulin injections (Álvarez-Buylla et al., 1959). This hypoglycemia is initiated after about one minute of latency.

2) This conditioned hypoglycemia is produced also in the totally pancreatectomized dog (Álvarez-Buylla and Carrasco Zanini, 1960).

3) The conditioned hypoglycemia is also obtained in dogs, two weeks after the spinal cord transection at the level of C 6, demonstrating that there is no spinal participation.

4) This conditioned hypoglycemia cannot be produced after vagotomy at the level of the diaphragm.

5) Injection of insulin in branches of the arterial celiac trunk produces a quick increase in the A. V. glycemia difference, but not if the vagal nerves have been cut. Stimulation of the central stump of the sectioned vagal nerves produces an increase in the difference of A. V. glycemia in a posterior extremity.

6) The afferent nerve fibers that follow the vagi trunks around the esophagus are the pathway for conducting afferent signals to the centers which produce the reflex hypoglycemia with very short latency.

Acknowledgements

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HEMATIC VALUES IN RESIDENTS AT ALTITUDES BETWEEN 1260 m AND 4515 m ABOVE SEA LEVEL

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RESULTS on chronic changes of hematic values with altitude differ among authors, who, with the exception of Fitzgerald [1], Hurtado et al. [2, 3] and Orías [4], limited their observations to one altitude level.

In trying to correlate the degree of chronic hypoxia induced by barometric depression to hematic changes, we felt necessary to determine the hematic parameters in permanent residents at different altitudes but with similar ethnic characteristics and nutrition habits. The results of such a study are presented in this paper.

MATERIALS AND METHODS

Except for a group of 49 female residents at 1260 m all the subjects were white or mixed indian males, whose physical characteristics are given in table 1. Excepting a group of draftees, the subjects were natives or permanent residents at the place for at least one year. The draftees were staying at 1260 m since 4 months previous to this study and according to their home sites were classified as follows: a) those who came from places located below 1500 m, b) those from places above 2000 m.

Venous blood obtained without stasis and transferred to a tube containing ammonium oxalate and potassium oxalate, was used for hemoglobin, hematocrit and red and white cells determinations. In all cases blood was drawn in the morning while the subjects were in fasting condition.

Hemoglobin: the sodium carbonate method was used and the readings taken on a Klett-Summerson photocolorimeter (540 mμ filter) previously calibrated by the oxygen capacity and the Wong ferrimetric methods. Values of the hemoglobin at 4515 m, already published in an earlier paper [5], were obtained with a Sahli hemoglobinometer calibrated by the oxygen capacity method. Determinations were made in duplicate.

Red and white cells: two counts were made and averaged.

Hematocrit: venous blood contained in Wintrobe tubes were centrifuged one hour at 3000 r.p.m.; duplicates were made.

Sedimentation rate: 2.6 ml of venous blood were drawn into a syringe containing 0.6 ml of a 3.8 per cent sodium citrate solution. The Westergreen method was employed with readings taken at the first hour.

Arterial oxygen saturation: it was

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determined in groups of residents at 1260, 2939, 3990 and 4515 m according to the technique described in an earlier paper (6).

The average of the barometric pressures at each altitude are given in table 1.

Results and discussion

The averages of the hematic values obtained at different altitudes are shown in table 2.

Hemoglobin: the average values of the draftees coming from low places and those of the older males, table 2, both residents in Jujuy (1260 m) do not differ between them, neither with those reported for sea level or low lands inhabitants (7-14). In 261 draftees quartered in Jujuy, Orías (4) found an average of 14.55 g per cent and in 23 officers 15.69 g per cent. He compared the average values found in draftees residents at various altitudes from sea level to 1260 m and observed a statistically significant increase, $p < 0.02$, at 1260 m.

Our draftees who came from above 2000 m showed values 2.7 per cent lower than those found in draftees from lowlands, the difference being statistically significant, $p = 0.01$.

In 25 male residents at 4540 m but who have been staying for six months to one year at sea level, Hurtado et al. (15) found hemoglobin values 8.1 per cent lower than those of the sea level residents.

In 49 female residents in Jujuy (1260 m) the average value, table 2, does not differ from those given at sea level or lowlands. Orías (11) found an average of 14.06 ± 0.06 g per cent in 94 girls living at 1260 m. Andresen and Mugrage (16) gave a mean value of 14.45 g per cent for 45 women residents at 1524 m. In 20 women living in Mendoza (747 m), Argentina, Sottano et al. (17) found an average of 13.84 ± 0.18 g per cent.

The mean values at 2078 m and 2939 m, table 2, were not statistically different but both were significantly greater than those found at 1260 m. The fact that subjects living at 2939 m did not show any increase of the hemoglobin values when compared to the residents at 2078 m can perhaps be explained by the nutrition conditions of the individuals. Actually, the average body weight of the latter was greater as it is shown in table 1.

The values at 3990 and 4515 m, table 2, were significantly different between them and to those found at lower altitudes, $p < 0.001$.

The average of 19.3 ± 0.60 found by Huff et al. (18) on 11 natives of Morococha (4540 m) is similar to that obtained by us in Mina Aguilar, at 4515 m above sea level.

In figure 1, the average of hemoglobin, red cells and hematocrit values of our subjects and their standard deviations are plotted against altitudes in kilometers. We included Talbott and Dill findings (19) on 6 residents for 2 to 14 years at 5334 m, in order to give a more complete picture of the hematic changes occurring within the levels where the permanent human residence is possible. Figures given by Fitzgerald (1) and Hurtado et al. (2, 3) are also included. Our hemoglobin results are slightly higher than those of Fitzgerald but significantly lower than values reported by Hurtado et al.

In figure 2 the hemoglobin values at 3990 and 4515 m are plotted against the percentages of the arterial oxygen saturation of the same individuals. It can be seen that the correlation between both parameters becomes statistically significant, $r = 0.575 \pm 0.125$, when the arterial oxygen saturation fells below 85 per cent.

When the hemoglobin averages found at various altitudes are plotted against

TABLE I

Physical characteristics of the subjects studied and average barometric pressure at each altitude

	Subjects	Place	Altitude (m)	Average barometric pressure (mm Hg)	Number of subjects	Age in years	Height (m)	Weight (Kg)
Males	Highlander draftees	Jujuy	1260	655	32	20	1,63 1.55-1.80 (*)	64.5 58-73 (*)
Males	Lowlander draftees	Jujuy	1260	655	44	20	1.67 1.57-184	64.8 53-85
Males	Civilians	Jujuy	1260	655	32	36.7 20-58 (*)	1.67 1.56-1.81	67.4 55-92
	Females	Jujuy	1260	655	49	29.5 21-44	1.55 1.42-1.74	60.6 43-97
	Males	Volcán	2078	595	32	34.4 21-53	1.68 1.59-1.76	80.1 63-101
	Males	Humahuaca	2939	536	45	29.8 16-68	1.64 1.53-1,82	63.7 51.86
	Males	Mina Aguilar	3990	475	15	37.8 24-56	1.65 1.63-1.78	61.0 53.70
	Males	Mina Aguilar	4515	446	84	33.8 21-54	1.62 1.51-1.79	64.3 52-88

(*) Range.

the arterial oxygen saturation averages, a nearly straight curve can be drawn (figure 3). On the other hand, in plotting hemoglobin values against altitudes a parabolic curve results which is alike to that obtained when arterial saturation percentages are plotted against altitudes (figure 4). The latter is an oxyhemoglobin dissociation curve giving the almost linear correlation between altitude and alveolar oxygen pressure.

Red Cells: The averages found in the draftees from low places and in the older male residents in Jujuy (1260 m) were lower than those found in the literature for similar or slightly lower altitudes [7-14].

In the draftees from highlands the red cell average was 2.2 per cent lower than in the draftees from lowlands; the difference was not statistically significant.

The average $4.46 \pm 0.046 \times 10^6$ found in 49 female residents at 1260 m was lower than those those given by others at sea level or low altitudes. Picón Reategui et al. [20] gave an average of $4.2 \pm 0.03 \times 10^6$ for 45 female residents in Lima (150 m), Perú. Andresen and Mugrage [16] obtained a mean value of 4.63×10^6 in 45 female residents at 1524 m.

The difference between the averages at 2078 and 2939 m was not statistically significant but both values were significantly greater than those at 1260 m. The averages at the higher altitudes, 3990 and 4515 m, were also greater than those at lower places.

Hurtado et al. [2] found an average of $6.15 \pm 0.07 \times 10^6$ in 32 permanent residents at 4540 m, a lower value, $p < 0.05$, than that obtained by us at 4515 m. On a later study [27] of 83 residents at the same altitude they found an average of $6.44 \pm 0.09 \times 10^6$. Barcroft and coworkers [21] found a mean value of 7.0×10^6 red cells in 15 residents at 4330 m.

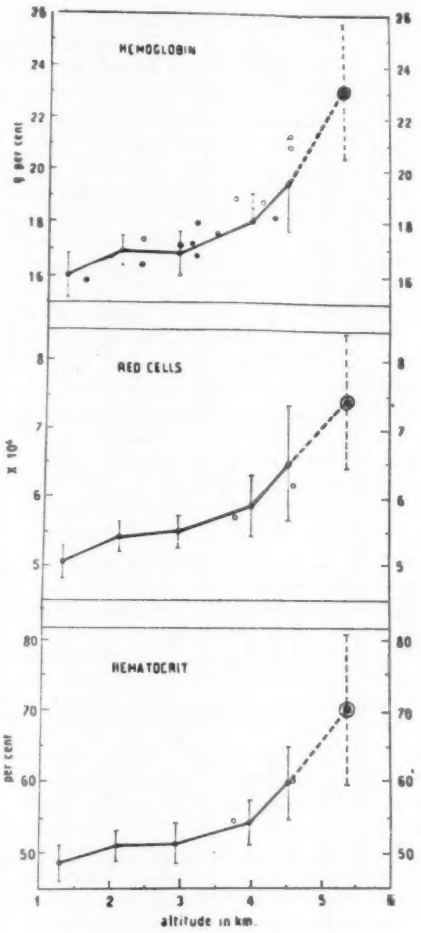

FIG. 1.—*Hemoglobin, red cells and hematocrit average values on healthy males living at high altitudes.*

Our data ± one standard deviation.
Data from Hurtado et al. (2).
Data from Fitzgerald (1).
Data from Talbott and Dill (19).

TABLE II

Observations on blood of healthy persons living at high altitudes

	Subjects	Place	Altitude (m)	Number of subjects	Hemoglobin (g/100 ml)	Red cells (x 10^6)	Hematocrit %	M. C. V. (μ^3)	M. C. H. ($\mu\mu$g)	M. C. H. C. (%)	Leucocytes (/mm^3)	Sedimentation rate (mm 1st hour)	Average art. oxygen. saturation (%)
Males	Highlanders draftees	Jujuy	1260	32	15.50 ± 0.12 (*)	4.927 ± 0.032	47.1 ± 0.37	95.6 ± 0.57	31.5 ± 0.23	32.9 ± 0.18	6650 ± 228	3.7 ± 0.41	—
	Lowlander draftees	Jujuy	1260	44	15.93 ± 0.11	5.037 ± 0.035	48.6 ± 0.41	96.5 ± 0.60	31.7 ± 0.19	32.8 ± 0.15	6966 ± 144	3.7 ± 0.33	95.1
	Civilians	Jujuy	1260	32	15.99 ± 0.19	0.015 ± 0.048	47.9 ± 0.53	95.3 ± 0.71	31.9 ± 0.28	33.5 ± 0.24	6539 ± 162	3.1 ± 0.34	95.1
	Females	Jujuy	1260	49	13.97 ± 0.137	4.461 ± 0.046	43.2 ± 0.51	96.5 ± 0.71	31.5 ± 0.19	32.7 ± 0.15	6600 ± 131	6.6 ± 0.40	—
	Males	Volcán	2078	32	16.88 ± 0.09	5.394 ± 0.043	50.8 ± 0.39	94.2 ± 0.38	31.3 ± 0.16	33.3 ± 0.17	—	—	—
	Males	Huma-huaca	2939	45	16.80 ± 0.12	5.468 ± 0.032	51.1 ± 0.42	93.3 ± 0,41	30.7 ± 0.15	33.0 ± 0.16	—	—	91.2
	Males	Mina Aguilar	3990	15	18.0 ± 0.27	5.840 ± 0.110	53.9 ± 0.80	92.5 ± 1.13	30.9 ± 0.42	33.4 ± 0.26	—	—	86.9
	Males	Mina Aguilar	4515	67	19.41 ± 0,22	6,460 ± 0.090	59.5 ± 0.58	92.4 ± 0.98	29.9 ± 0.26	32.7 ± 0.16	6744 ± 198	0.7 ± 0.10	82.8

(*): Standard error of the mean.
M. C. V.: Mean Corpuscular Volume.
M. C. H.: Mean Corpuscular Hemoglobin.
M. C. H. C.: Mean Corpuscular Hemoglobin Concentration.

Hematocrit: the hematocrit average of the draftees from lowlands and that of the older males residents at 1260 m were not statistically diferent, table 2, but were higher than those found at sea level ([12-14]). Nevertheless they do not differ significantly from the values given by Fonio and Moglia ([10]) for males living in Tucumán (427 m) and by Sottano et al. ([17]) in 29 young men residents in Mendoza (747 m), Argentina.

After 4 months of residence at 1260 m the draftees who came from high places showed hematocrit values 3.1 per cent lower than in those who came from lowlands, table 2, the difference being statistically significant, $p < 0.01$.

The average of 43.2 $\pm$ 0.15 corresponding to female residents in Jujuy (1260 m) was greater than those found by many authors at lower altitudes or sea level ([4, 10, 12, 20]). Nevertheless, it agrees with the value of 43.2 $\pm$ 0.51 given by Sottano et al. ([17]) for 19 female residents at 747 m. Andersen and Mugrage ([16]) gave an average of 43.2 per cent in 45 female residents at 1524 m.

As it happened with the other hematic parameters it did not exist any significant difference between the averages of the hematocrit of the residents at 2078 and 2939 m. With this exception, in all the other cases the hematocrit readings were significantly greater as the subjects lived at a higher place. Our values at 4515 m agree with those given by Hurtado et al. ([2, 27]) at 4540 m.

Mean corpuscular hemoglobin (M. C. H.) and mean corpuscular hemoglobin concentration (M. O. H. C.): the M. C. H. averages of male and female residents at 1260 m, table 2, were significantly higher than most of those given at sea level, the difference being due to the lower red cell counts found in our subjects. Picón Reategui et al. ([20]) obtained an average of 31.8 $\pm$ 0.20 $\mu\mu$g in 150 female residents in Lima (150 m), a figure similar to that found by us at 1260 m, table 2.

The M. C. H. average felt progressively with the increasing altitude, the difference between 1260 and 4515 m values being highly significant, $p < 0.001$.

The averages of the M. C. H. and

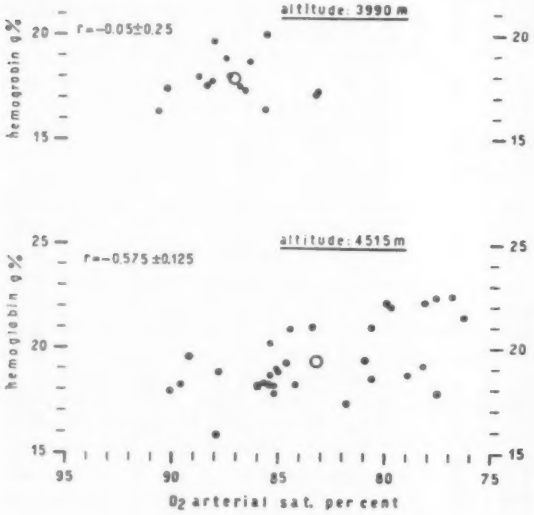

FIG. 2. — *Relationship between individual values of hemoglobin and arterial oxygen saturation of healthy males living at 3990 m and 4515 m above sea level.*

Individual values.

Average.

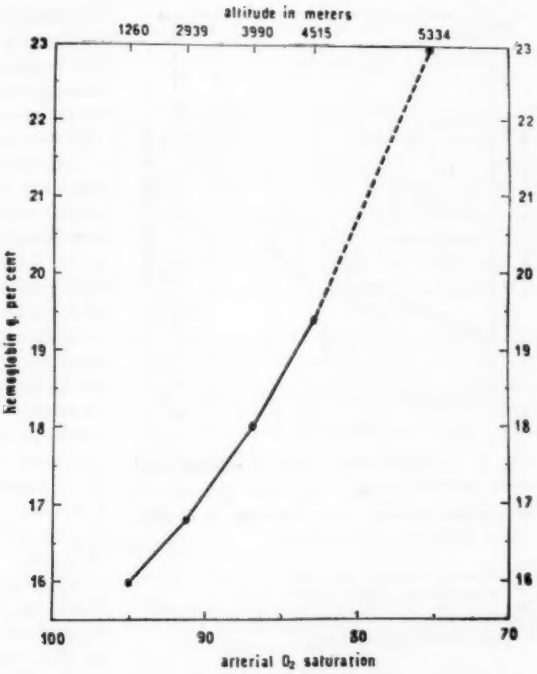

FIG. 3. — *Relationship between arterial oxygen saturation and hemoglobin average values of healthy males living at high altitudes.*

Our data.

Data from Talbott and Dill (19).

M. C. H. C. of our residents at 4515 m were significantly lower than those given by Hurtado et al. (2) the difference being due to the lower hemoglobin values and to the higher red cell counts of our subjects.

Mean corpuscular volume (M. C. V.): the M. V. C. averages found at 1260 m were in all cases higher than those found by others at lower places or at sea level. Picón-Reategui et al. (20) gave an average of 94.1 $\pm$ 0.50 μ^3 for 150 female residents at 150 m, figure significantly lower, $p < 0.01$, than ours at 1260 m, table 2. Although the M. C. V. showed a trend to fall with increasing altitudes, such a decrease was only probably significant at 3990 and 4515 m, $p = 0.05$ and $p = 0.02$ respectively, when compared to the values at 1260 m.

Hurtado et al. (2) found the following mean values: 91.3 $\pm$ 0.23 (*) at 150 m, 95.2 $\pm$ 0.58 (*) at 3730 m and 97.5 $\pm$ 0.75 (*) at 4540 m. This last figure was significantly higher, $p < 0.001$ than ours at 4515 m (table 2). However, later studies made by the same authors (27) on a greater number of subjects gave results quite similar to those of the present study.

Leucocytes: the leucocyte averages of the residents at 1260 m and 4515 m, table 2, did not show any significant deviation from the normal sea level values. Hurtado et al. (2) found a mean value of 6900 $\pm$ 144 (*) per mm^3 in 32 residents at 4540 m and 6500 $\pm$ 155 (*) in 40 residents at 3730 m.

Sedimentation rate: values within the normal limits at sea level were found in residents at 1260 m. The

(*) Probable error.

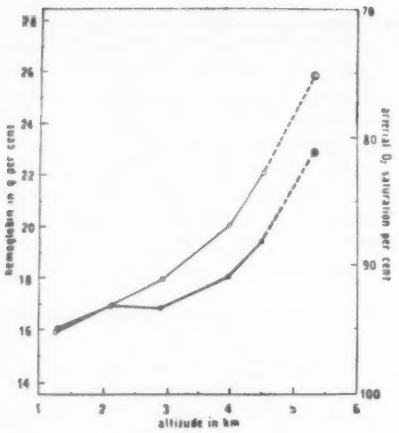

FIG. 4. — *Relationship between altitude and mean arterial oxygen saturation* ○—○ *or hemoglobin average values* ●—● *in healthy males living at high altitudes.*

◉ *Arterial oxygen saturation average in Talbott and Dill's subjects* (19).
◎ *Hemoglobin average value in Talbott and Dill's subjects* (19).

decrease observed at 4515 m and already published in a previous paper (5) was highly significant, $p < 0.001$, when compared with the 1260 m average.

Our results on the hematic changes in permanent residents between 1260 and 4515 m above sea level, agree with the very well known fact of a progressive increase of the absolute and relative quantities of hemoglobin and red cells with increasing altitude.

Comparing our results at 1260 m with those obtained by other authors at lower levels, no clear increases of the hematic values of men and women living at that altitude are apparent. Since we have not made any determination on sea level residents we can not draw any conclusion, especially because in the case it would exist, the difference should not be particularly noticeable. In 1930, Orías (4) observed an increase of 0.42 g per cent in the hemoglobin average of draftees residents at 1260 m when compared to the sea level values.

On the other hand, the increase of the red cells, hemoglobin and hematocrit averages are quite evident in residents at 2078 m showing that even mild chronic hypoxia will stimulate the bone marrow. Such changes are still greater beyond 3000 m.

The changes with altitude of the number of the red cells and amount of hemoglobin tend to be parallel, although the statistically significant decrease of the mean corpuscular hemoglobin at high altitude shows that such parallelism is not a perfect one. Likewise, a not so evident diminution of the mean corpuscular volume also ocurred.

The fact that in the same individual the hemopoietic response to changes in altitude shapes a curve similar to that of the arterial oxygen saturation supports the view that the hemopoietic function of the bone marrow would be under the control of tissular oxygen pressure. In fact and if as stated by Korner (22), the hemopoietic function should be under the control of the arterial oxygen pressure, the stimulation curve produced by hypoxia should approach a straight line like the one obtained plotting arterial or alveolar oxygen pressure against altitude. This point of view agrees with the existing correlation between the erythropoietic activity and the degree of clinical or experimental anemia (22), even if in these cases the arterial oxygen pressure remains normal. Consequently, it is the oxyhemoglobin content of the blood and not the arterial oxygen pressure that controls the hemopoietic activity of the bone marrow. This is easy to understand since it is the oxygen content of the arterial blood and not its

oxygen pressure which regulates the tissue levels of oxygen. The probable formation of erythropoietin in the kidney under hypoxemic or anemic stimulation ([24, 25]) strengthens this point of view. In a recent work from our laboratories ([26]), significant amounts of erythropoietin were found in the urine of residents at 3990 and 4515 m above sea level, particularly during the first week after arrival to altitude.

The increase of hemoglobin should be considered as a secondary adaptive mechanism coming into action only when the fall of the oxygen pressure reaches the tissue level, and consequently after the respiratory mechanisms are already stimulated by arterial hypoxemia.

Summary

Hemoglobin, red cell number and hematocrit values were determined on permanent residents at 1260, 2078, 2939, 3990 and 4515 m above sea level.

The number of red cells, hemoglobin and hematocrit values increased with altitude from 2078 to 4515 m. At 1260 m no significant increase was found.

The mean corpuscular hemoglobin and the mean corpuscular volume decreased progressively between 1260 and 4515 m while no significant changes were observed in the mean corpuscular hemoglobin concentration.

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LA FIBRILACIÓN VENTRICULAR EN EL CORAZÓN PERFUNDIDO DEL PERRO (*)

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Una de las características de la fibrilación ventricular en el corazón de los mamíferos es que puede ser producida por estímulos únicos cuando éstos son aplicados durante el período refractario del ciclo de recuperación. Los estudios de Wiggers (11), Wegria y Wiggers (10), Orías (8), Brooks (2) y Hoffman *et al.* (6, 7) han precisado la ubicación temporal de esta fase del período refractario y le han dado el nombre de período vulnerable. Además, Hoffman *et al.* (6, 7) han señalado que el período vulnerable corresponde en el tiempo a los llamados "dips" (Orías *et al.*) (10) de la curva de recuperación de la excitabilidad ventricular, y que para que se produzca fibrilación es necesario que los estímulos de prueba sean de intensidad superior al umbral para ese momento del ciclo. Por otro lado, los estímulos de intensidad supraumbral pero inferior a la necesaria para provocar fibrilación darían origen a actividad repetitiva.

García Ramos y Miledi (4) encontraron que para producir fibrilación por aumento gradual de la frecuencia, era necesario utilizar estímulos de intensidad cuatro a cinco veces el valor umbral.

La mayor parte de los trabajos sobre este tema han sido efectuados en corazones "in situ". La complejidad de variables impuesta por la presencia del sistema nervioso y de las glándulas suprarrenales, adicionada a las modificaciones en la circulación coronaria inherentes a un corazón que está irrigándose a sí mismo, dificultan el análisis de la fibrilación ventricular. Por lo tanto se creyó conveniente estudiar dicho fenómeno en una preparación experimental que careciera de los inconvenientes señalados.

Método

Se utilizaron corazones de perros adultos que fueron perfundidos por otro perro de acuerdo con la técnica descrita originalmente por Heymans y Kochman (5) modificada por García Ramos, Alanís y Rosenblueth (3). La sangre arterial procedente del corazón donador, se hizo llegar al corazón perfundido a temperatura constante (36° C ± 0.5° C). La presión con que esta sangre llegaba al sistema coronario (presión de perfusión), fué medida por

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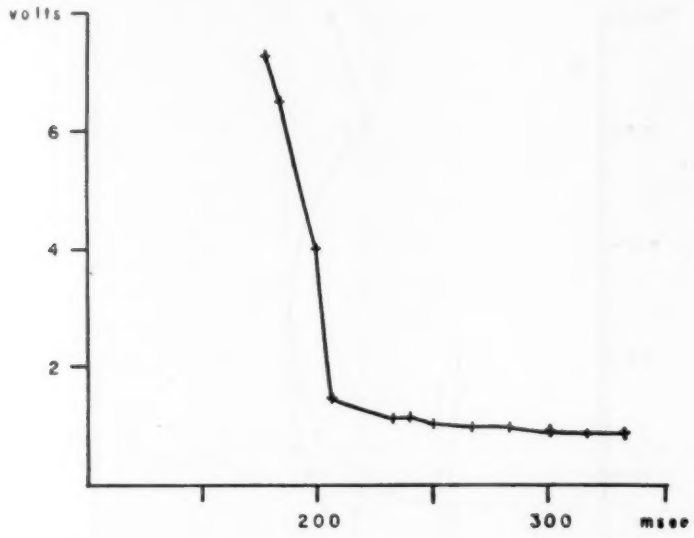

FIG. 1.—*El ciclo de recuperación de la excitabilidad ventricular obtenido con frecuencia basal lenta. En esta gráfica la curva de recuperación de la excitabilidad se obtuvo con una frecuencia basal de 2.4/seg; sus diferentes puntos corresponden al valor umbral del estímulo de prueba para ese momento del ciclo cardíaco. En cada punto se aumentó la intensidad del estímulo hasta veinte o más veces el valor umbral. En ninguno de los corazones estudiados en esta forma se produjo fibrilación. Abscisas, intervalo E_1-E_2 en milisegundos. Ordenadas, intensidad del estímulo de prueba (E_2) en volts.*

medio de un manómetro de mercurio intercalado en la proximidad de la cánula arterial. Los resultados que se describen corresponden a corazones cuya presión de perfusión fué de 90-110 mm Hg.

El nodo seno-auricular fué destruído por machacamiento y el ventrículo fué estimulado directamente por medio de pulsos rectangulares provenientes de dos estimuladores (Grass, modelo S 4). Los estimuladores estaban acoplados de manera que uno de ellos producía un primer estímulo (E_1) con el cual se estimuló al ventrículo a una frecuencia determinada (frecuencia basal); con el otro estimulador se obtenía un segundo estímulo (E_2), que se repetía en el curso de una determinación a intervalos no menores de 15 seg y cuya duración, intensidad y separación con respecto a E_1, podían ser controladas independientemente. La duración de ambos estímulos fué en todos los casos de 2 mseg. Los pulsos fueron separados de tierra por medio de unidades aisladoras y aplicados en la base de la superficie ventricular derecha por medio de un par de electrodos de pinza.

La actividad eléctrica del músculo ventricular, fué registrada por medio de un par de electrodos de pinza con 2 cm de distancia interelectrodal colocados a 2 cm de distancia de los electrodos de estímulo y conectados a un galvanómetro de bobina móvil.

Para desfibrilar al corazón se utilizó el método de Hooker *et al.* [8] aplicando al ventrículo corriente alterna de 50 ciclos y 115 volts durante 0.5 seg.

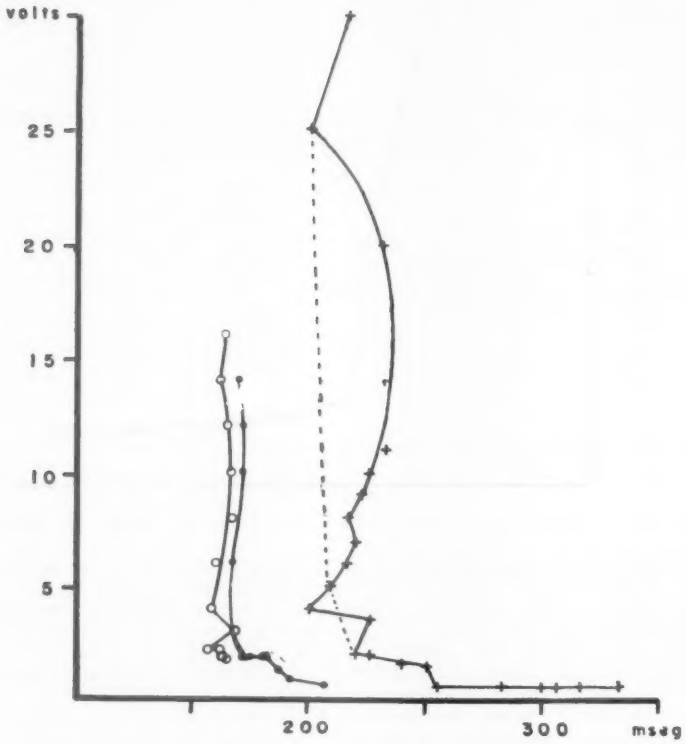

FIG. 2. — *La curva de recuperación de la excitabilidad y el fenómeno de la "no respuesta". Las curvas de esta gráfica fueron obtenidas determinando los intervalos mínimos E_1-E_2 para los estímulos de prueba de intensidad fija. La curva de las cruces fué obtenida con una frecuencia de estimulación basal de 2.5/seg; la curva de puntos con una frecuencia de 3.5/seg y la curva de los círculos con una frecuencia de 4.1/seg. La curvatura de concavidad izquierda que se observa en la línea de cruces, parece ser característica del ciclo de recuperación cuando se estimula el ventrículo con frecuencias basales lentas; al aumentar la frecuencia basal la curvatura se atenúa y llega a desaparecer. La línea interrumpida corresponde a la curva obtenida por el método de determinar umbrales; la zona comprendida entre esta última y la concavidad de la línea de cruces corresponde a la región en la que se observó el fenómeno de "no response".*

RESULTADOS

1. *El ciclo de recuperación con frecuencias basales lentas.* — Los resultados que en seguida se describen fueron obtenidos en corazones cuya frecuencia basal de estimulación fué inferior a 3/seg y en los cuales la curva de recuperación de la excitabilidad fué estudiada determinando la intensidad umbral de E_2 para los diferentes momentos del ciclo cardíaco.

La gráfica de la figura 1 corresponde a los resultados obtenidos en uno de estos experimentos; en este caso la frecuencia basal fué de 2.4/seg y el inter-

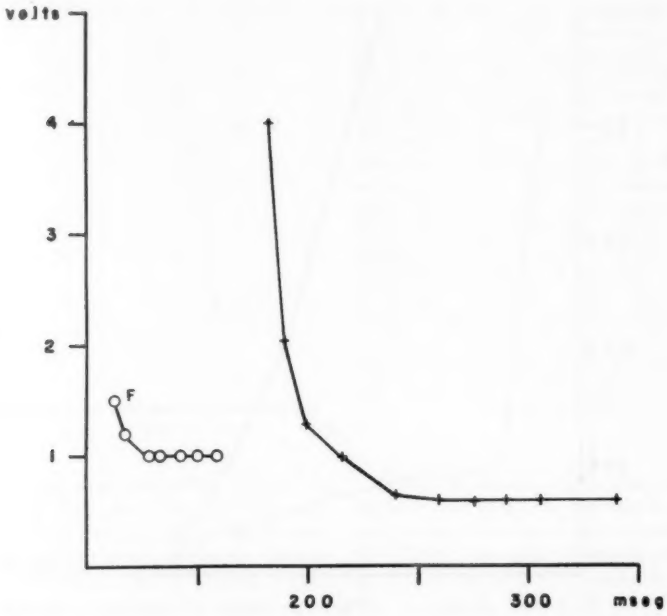

FIG. 3.—*La curva de recuperación de la excitabilidad ventricular y su relación con la fibrilación. Ambas curvas fueron obtenidas por el método de determinar umbrales. La línea de los círculos fué obtenida con una frecuencia basal de 4.5/seg; el último círculo marcado con F, corresponde al intervalo E_1-E_2 en el cual el estímulo de prueba produjo fibrilación. La línea de las cruces corresponde a una frecuencia basal de 2.4/seg. Comparando esta última con la obtenida a frecuencia basal alta, se pone de manifiesto el acortamiento del período refractario provocado por el aumento de la frecuencia y la posibilidad de alcanzar intervalos E_1-E_2 adecuados para provocar fibrilación. Coordenadas como en la figura 1.*

valo mínimo E_1-E_2, al cual se produjo respuesta ventricular (R_2) al estímulo de prueba, fué de 177 mseg. El valor promedio de este intervalo mínimo E_1-E_2 para los experimentos con frecuencias basales lentas (1.2-3.0/seg) fué de 170 mseg, con valores extremos de 140 y 195 mseg. Para las frecuencias más cercanas a 3/seg correspondieron los intervalos E_1-E_2 más breves.

Después de haber obtenido la curva de recuperación se procedió a estudiar cada uno de los intervalos E_1-E_2 de la misma curva, pero ahora con estímulos de prueba cuya intensidad fué hasta 10 ó 20 veces el valor umbral.

En ninguno de estos corazones estimulados con frecuencia basal lenta se obtuvo fibrilación ventricular al determinar la curva de recuperación de la excitabilidad, ni al aplicar estímulos de prueba de intensidad supraumbral. En algunos casos pudo observarse la producción de respuestas repetitivas al aplicar estímulos E_2 umbrales o supraumbrales. La aparición de estas respuestas estuvo condicionada por el momento del ciclo cardíaco en el que fueron aplicados los estímulos E_2. Este momento fué variable para los distintos corazones estudiados, en general se le pudo ubicar entre los 150 y los 200

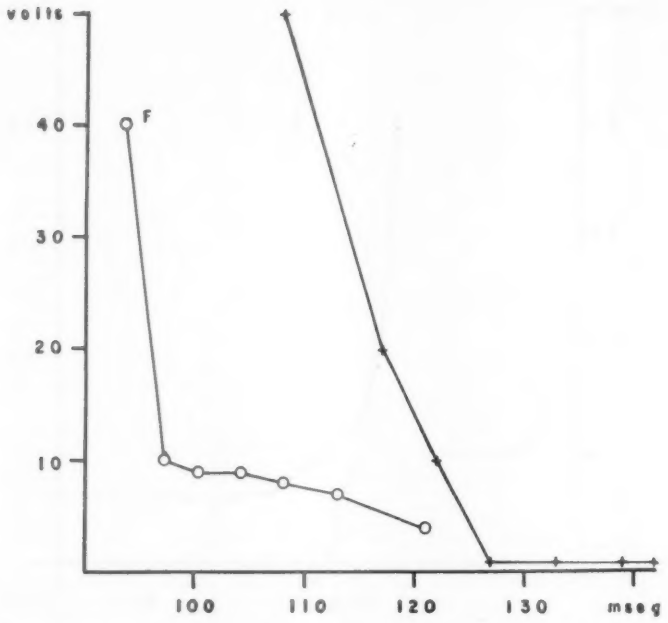

FIG. 4. — *La curva de recuperación de la excitabilidad a frecuencias basales altas y su relación con la fibrilación ventricular. La curva de las cruces fué obtenida con frecuencia basal de 5.2/seg; no se produjo fibrilación. Al aumentar la frecuencia basal a 5.4/seg (curva de los círculos), se alcanzó un intervalo E_1-E_2 más breve en el cual el estímulo de prueba provocó fibrilación ventricular. En estas gráficas se pone de manifiesto que es necesario alcanzar un determinado valor de intervalo E_1-E_2 para que se produzca la fibrilación.*

mseg a partir del principio del ciclo y abarcó 10 ó 20 mseg del mismo.

Cuando se aplicaron estímulos de prueba de intensidad supraumbral pudo observarse, que para un mismo intervalo E_1-E_2 las intensidades altas con respecto al umbral no produjeron respuesta propagada, en tanto que una intensidad menor, era capaz de producirla. Para precisar las condiciones en que se presentaba este fenómeno se hizo el estudio de la curva de recuperación de la excitabilidad buscando el intervalo mínimo E_1-E_2 al cual un estímulo de prueba de intensidad fija, producía una respuesta propagada. Las intensidades de E_2 utilizadas abarcaron desde el valor umbral para un momento tardío en el ciclo de recuperación hasta 150 volts.

La curva de recuperación de la excitabilidad obtenida por este método, se caracteriza por presentar en su rama vertical una curvatura de concavidad izquierda. Este hecho se encuentra ilustrado en la gráfica de la figura 2 por la línea continua de cruces. La línea interrumpida corresponde a la curva de recuperación obtenida en el mismo corazón y a la misma frecuencia basal (2.5/seg), por el método de determinar intensidades umbrales para los diferentes momentos del ciclo cardíaco. Los estímulos de prueba que por sus coor-

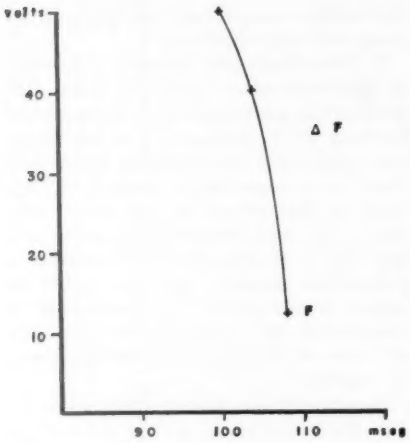

FIG. 5. — *Relación entre el intervalo E_1-E_2 necesario para producir fibrilación y el ciclo de recuperación de la excitabilidad. Los puntos de estas gráficas fueron obtenidos por el método de determinar intensidades umbrales, mas ahora se principió por los intervalos más breves (inferiores a 100 mseg). El símbolo* △ *marcado con F indica que esa fué la primera respuesta R_2 que se pudo obtener con frecuencia basal de 5.1/seg y que provocó fibrilación. Al aumentar la frecuencia basal a 5.7/seg se pudieron obtener intervalos E_1-E_2 más breves y cuando el intervalo se aproximó a 110 mseg se produjo fibrilación (última cruz de la derecha). Nótese que es posible producir respuestas R_2 cuando los estímulos de prueba se aplican a intervalos más cortos que aquel en el que se produjo la fibrilación. Coordenadas como en la figura 1.*

denadas quedaron colocados dentro de la zona limitada por la línea interrumpida y la concavidad de la línea continua, no produjeron respuesta propagada. Esta concavidad se atenúa y aun llega a desaparecer cuando se aumenta la frecuencia basal (línea de puntos 3.5/seg y línea de círculos 4.5/seg de la figura 2).

2. *El ciclo de recuperación y la fibrilación ventricular con frecuencias basales rápidas.* — En estos experimentos la frecuencia basal promedio fué de 5/seg, con valores extremos de 4/seg y 6.5/seg. La curva de recuperación de la excitabilidad fué estudiada por el método de determinar intensidades umbrales para los estímulos de prueba aplicados en diferentes momentos del ciclo. En estas condiciones se llegó a un intervalo E_1-E_2 en el cual, al alcanzar el estímulo de prueba la intensidad umbral, se produjo la fibrilación ventricular. La curva de recuperación de la excitabilidad se encontró desplazada a la izquierda y, en consecuencia, los intervalos E_1-E_2 mínimos que provocaron respuesta y a los cuales se produjo la fibrilación, fueron más breves que los intervalos mínimos obtenidos con frecuencias basales lentas.

Estos resultados se ilustran en la gráfica de la figura 3. La curva de recuperación obtenida a una frecuencia basal de 4.5/seg está representada por la línea de círculos; el círculo de la extrema izquierda marcado con F, indica que a ese intervalo y con un estímulo de prueba de intensidad umbral se produjo fibrilación ventricular. La línea de cruces fué obtenida previamente en el mismo corazón y corresponde al ciclo de recuperación para una frecuencia basal de 2.4/seg.

En algunos de estos corazones se encontró que aun cuando el ciclo de recuperación permitía alcanzar intervalos E_1-E_2 inferiores a 120 mseg no se produjo fibrilación. Sin embargo, al aumentar más la frecuencia basal, la curva de recuperación se desplazó a la izquierda y en estas condiciones, fué posible alcanzar un intervalo E_1-E_2 en el cual el estímulo de prueba provocó la fibrilación ventricular. Un ejemplo de estos resultados está ilustrado en la figura 4. La curva de las cruces corresponde al ciclo de recuperación para una frecuencia basal de 5.2/seg. En este caso el intervalo mínimo E_1-E_2 al cual se obtuvo respuesta R_2, fué de 108 mseg sin que se produjera fibrilación; la cur-

va de los círculos corresponde al ciclo de recuperación del mismo ventrículo con una frecuencia basal de 5.4/seg, el intervalo mínimo E_1E_2 fué de 93 mseg y en este caso el estímulo E_2 de intensidad umbral provocó fibrilación.

Con objeto de saber si era posible obtener respuesta a estímulos de prueba aplicados a intervalos E_1-E_2 más cortos que aquellos a los que se producía la fibrilación, se procedió como sigue. En corazones en los que se había producido fibrilación se interrumpió esta fibrilación y después de 15 min se aumentó la frecuencia basal con objeto de acortar más el período refractario. En seguida se aplicó el estímulo de prueba, buscando intensidad umbral, a intervalos E_1-E_2 más breves que aquel con el cual el ventrículo fibriló. En los corazones en que no se había producido fibrilación, se procedió a determinar la curva de recuperación de la excitabilidad, también buscando intensidades umbrales, más ahora a partir de intervalos E_1-E_2 muy breves (inferiores a 100 mseg). En ambos casos fué posible obtener respuestas R_2, sin fibrilación, para intervalos E_1-E_2 más cortos que aquellos a los que el ventrículo fibrilaba.

Los resultados obtenidos en uno de estos experimentos están ilustrados en la figura 5. El corazón en estudio fué estimulado con frecuencia basal de 5.1/seg. Se procedió de acuerdo con la segunda técnica descrita arriba y la primera respuesta que se obtuvo fué la fibrilación ($\triangle$F); se aumentó la frecuencia basal a 5.7/seg y se obtuvo la curva marcada con cruces, en el último punto (108 mseg) se produjo fibrilación.

El valor promedio de intervalo E_1-E_2 al cual se produjo la fibrilación en todos los corazones estudiados, fué de 108 mseg con valores extremos de 150 y 91 mseg. El margen del intervalo E_1-E_2 dentro del cual se produjo la fibrilación comprendió en general 20 mseg del ciclo cardíaco.

3. *Resultados del aumento gradual de la frecuencia.* — A fin de saber si la fibrilación provocada por el aumento gradual de la frecuencia de estimulación requería estímulos cuya intensidad fuera muy superior al umbral, se procedió a determinar la intensidad umbral para una frecuencia baja (1 a 2 por seg) y en seguida, se determinó la frecuencia máxima que era capaz de seguir el ventrículo en función de la intensidad del estímulo, considerando el valor de la intensidad umbral como la unidad.

Los resultados que se obtuvieron están ilustrados en la gráfica de la figura 6. Los puntos de la curva, excepto el último, corresponden a la frecuencia máxima seguida por el ventrículo antes de alternar; el último punto, marcado con F, corresponde a la frecuencia máxima alcanzada inmediatamente antes de que el ventrículo fibrilara. En todos los corazones estudiados fué posible obtener fibrilación con estímulos cuya intensidad fué inferior a dos veces el valor umbral. El valor promedio fué de 1.6 unidades umbral con extremos de 1.8 y 1.3. La frecuencia máxima promedio inmediatamente previa a la fibrilación fué de 6.6/seg con valores extremos de 8/seg y 5.8/seg.

Discusión

A. *El método.*— La preparación experimental empleada permite excluir las variables impuestas por el sistema nervioso y las glándulas suprarrenales; además, proporciona la ventaja de que la circulación coronaria del corazón en estudio se altera en grado mínimo durante los períodos de fibrilación ([8]). En la mayoría de los corazones estudiados, pudo observarse que después de dos o tres episodios de fibrilación la facilidad para provocar este fenómeno era mayor, perdiéndose la relación entre

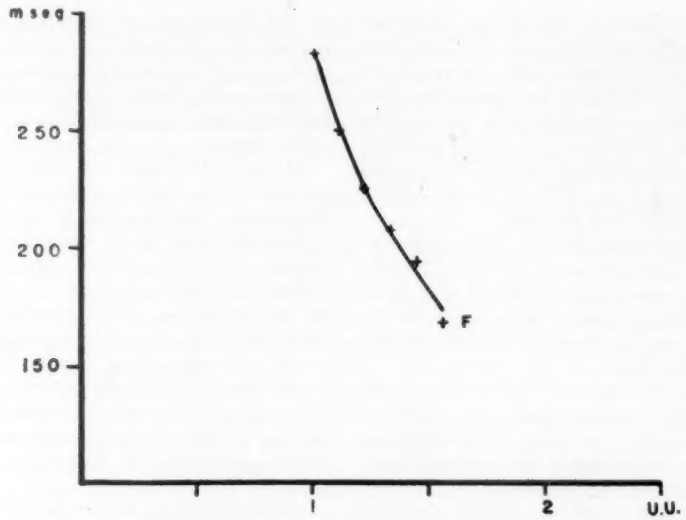

FIG. 6. — *Relación entre la intensidad del estímulo y la fibrilación ventricular provocada por el aumento gradual de la frecuencia. Todos los puntos excepto el último de la derecha, indican la frecuencia máxima que siguió el ventrículo antes de alternar, en función de la intensidad del estímulo correspondiente. El último punto de la derecha marcado con F, indica la frecuencia máxima inmediatamente previa a la fibrilación. Abscisa, intensidad del estímulo en unidades umbral (umbral determinado a frecuencia inferior a 2/seg). Ordenada, longitud del ciclo (recíproca de la frecuencia), previa a la alternancia o a la fibrilación, en milisegundos.*

intervalo E_1-E_2 y fibrilación. Teniendo en cuenta lo anterior, los resultados que han sido descritos corresponden a los dos o tres primeros episodios de fibrilación de cada corazón estudiado.

B. *El ciclo de recuperación en los corazones estimulados con frecuencias basales lentas.* — Entre las conclusiones referentes a la producción de la fibrilación ventricular por aplicación de estímulos únicos de prueba, se encuentran las de Wiggers y Wegria [15] quienes afirman que la fibrilación ventricular por estímulos únicos se produce cuando éstos se aplican durante la parte tardía de la sístole precedente. Por otra parte, Hoffman *et al.* [6] han relacionado la producción de la fibrilación ventricular con la existencia de ciertas alteraciones en la forma de la curva de recuperación de la excitabilidad (dips), afirmando que para que los estímulos de prueba puedan producir fibrilación, es necesario que sean aplicados en el momento del ciclo de recuperación correspondiente al "dip" y con intensidad superior a la umbral.

Los resultados descritos en la primera sección de este trabajo, ponen de manifiesto que cuando la frecuencia basal de contracción ventricular es lenta, no es posible producir fibrilación por estímulos de prueba, no obstante que éstos sean aplicados durante la última fase de la sístole precedente; por otra parte, aun cuando en la preparación experimental estudiada no fué posible poner en evidencia los "dips" de la curva de recuperación, los estímulos de prueba de intensidad 10, 20 o más veces el valor umbral, aplicados en diversos momentos del ciclo tam-

poco provocaron fibrilación ventricular. Puede concluirse por lo tanto, que en el corazón perfundido no se puede producir fibrilación por estímulos únicos cuando la frecuencia de contracción es lenta (inferior a 3 por segundo), independientemente del momento del ciclo en que se aplique el estímulo y de la intensidad de éste.

El fenómeno de la "no response" descrito por Orías *et al.* (11) y Brooks *et al.* (1), puede ser explicado si se tiene en cuenta la forma de la curva de recuperación de la excitabilidad ilustrada en la figura 3. En esta gráfica se muestra que un estímulo de prueba de intensidad umbral para un intervalo determinado (5 volts, 210 mseg) no produce respuesta a ese mismo intervalo si su intensidad se aumenta, dentro de ciertos límites, debido a que la curvatura de concavidad izquierda, que parece ser característica del proceso de recuperación, lo coloca en una zona de inexcitabilidad. Un fenómeno semejante, por lo que se refiere a la forma de la curva de recuperación, fué observado en las fibras nerviosas por Miledi (observaciones no publicadas).

C. *La fibrilación ventricular en corazones estimulados con frecuencias basales altas.* — Los resultados descritos en la segunda sección ponen de manifiesto que cuando la frecuencia basal es alta (4 o más por segundo) puede producirse fibrilación ventricular cuando se aplica un estímulo de prueba a un intervalo E_1-E_2 adecuado. El hecho de que estímulos aplicados a intervalos E_1-E_2 más breves o más largos que el adecuado no produzcan fibrilación, permite afirmar que se trata de un intervalo crítico que por estar localizado dentro del período refractario relativo, y por ser crítico, corresponde al período vulnerable descrito por Wiggers (14), Wiggers y Wegria (15) estudiado por Orías *et al.* (11) para la aurícula y por Hoffman *et al.* (6, 7) para el ventrículo. Sin embargo, es importante señalar que en los experimentos descritos en este trabajo, la fibrilación se produjo con estímulos de intensidad umbral, lo cual produce una diferencia importante con los resultados comunicados por los autores citados.

El hecho de que no fuera posible producir fibrilación ventricular con frecuencias basales lentas puede ser explicado si se tiene en cuenta que debido al acortamiento del período refractario provocado por el aumento de la frecuencia (Rosenblueth) (12) el intervalo E_1-E_2 al cual se produjo fibrilación con frecuencias rápidas fué más breve que el intervalo mínimo E_1-E_2 obtenido con frecuencias lentas; por lo tanto, en estas últimas condiciones no pudo alcanzarse el intervalo crítico para la fibrilación.

Sin embargo, el hecho de que en algunos corazones estimulados con frecuencias lentas se pudieran obtener respuestas R_2 a intervalos E_1-E_2 inferiores a 150 msegs y no obstante no se provocara fibrilación, permite suponer que además del intervalo crítico es necesario la presencia de otro factor que aparecería cuando la frecuencia de estimulación es rápida.

D. *La fibrilación ventricular producida por el aumento gradual de la frecuencia.* — Los resultados descritos en la última sección de este trabajo ponen de manifiesto que en el corazón perfundido se puede provocar fibrilación ventricular al aumentar gradualmente la frecuencia, con estímulos cuya intensidad no es muy diferente de la umbral (menos de dos veces el valor umbral).

Resumen

En corazones de perro aislados y perfundidos por la sangre de un animal donador, se estudió la fibrilación ventricular producida por la aplicación de estímulos únicos de prueba.

Cuando el ventrículo fué estimula-

do con frecuencias basales lentas (inferiores a 3/seg) los estímulos de prueba fueron ineficaces para provocar fibrilación, independientemente del momento del ciclo de recuperación en que fueron aplicados y de su intensidad.

La forma de la curva de recuperación de la excitabilidad ventricular, determinada por el procedimiento de encontrar el intervalo mínimo al que diferentes estímulos de prueba de intensidad fija produjeron respuesta propagada, se caracterizó por presentar en su rama vertical una curvatura de concavidad izquierda. Con base en este hecho se propone una explicación para el fenómeno descrito en la literatura con el nombre de "no response".

Cuando el ventrículo fué estimulado con frecuencias basales altas (4 o más por segundo) se pudo provocar fibrilación con estímulos de prueba de intensidad umbral, cuando éstos fueron aplicados en un intervalo adecuado del ciclo de recuperación. Estímulos aplicados antes o después de este intervalo, fueron ineficaces para hacer fibrilar al ventrículo, aun cuando provocaron respuestas propagadas.

Para producir fibrilación ventricular por el aumento gradual de la frecuencia de estimulación, se necesitaron estímulos cuya intensidad fué menor de dos veces el valor umbral.

Los autores agradecen a los doctores A. Rosenblueth y J. Alanís, las sugestiones recibidas durante la elaboración del presente trabajo y las correcciones hechas al manuscrito.

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ADMINISTRATION OF PELLETS OF MORPHINE TO MICE; ABSTINENCE SYNDROME

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As it is known, when a substance is administered to the organism a high blood concentration of it is rapidly obtained, which decreases slowly afterwards. On the contrary, when the compound is compressed and introduced as a pellet, there is a slow absorption and the blood level is maintained for long periods ([8]). The absorption rate of the pellet depends on the solubility of the drug in the interstitial fluid. The more insoluble the substance is the more slowly it is absorbed ([7]). Thus, it is possible to flood the organism with a drug for a long time.

It was thought interesting to study the reaction when morphine is introduced as a pellet, because in this case the organic adaptation may be different to that observed when the substance is injected in solution. On the other hand, the continuous arrival of morphine to its receptors, is a new approach to the study of phenomena of tolerance and physical dependence.

METHOD

Adult male and female white mice (Catholic University's strain) were employed. Special care was taken to use animals of the same weight and to administer homologous quantities of drugs.

General attitude was studied. Position, excitation, tranquility, depression, sleep, catatony, etc. were investigated in every animal and in the group. The tail and the eye position were observed. Pain thereshold was analized by Jacob and Blosowsky's ([6]) modification of the Woolfe and Mac-Donald's hot plate method ([11]); the muscular tension was appreciated by Frommel's technique ([3]). Furthermore, the animals were forced to resolve a "complex test": they were put on the surface of a column (26.5 by 26.5 cm of base and 21 cm of height) attached to another equal column by a cylinder of wood (100 cm of length and 0.9 cm of diametre); at the base of the second column the cage where they used to live was placed. Three periods were distinguished in the test: 1) time taken by the animal to leave the first surface; 2) time taken to cross the cylinder of wood and 3) time taken to arrive to the cage after crossing the bridge.

In some opportunities the movements of the mice were recorded. Groups of 4 mice were placed on a surface which was set down on a sensible spring; the surface was attached to the short end of a level which recorded the movements at a kymograph.

All the tests were performed in the

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most similar conditions dealing with time, light, room temperature, etc.

Drugs used were: Morphine hydrocloride transformed into the base and compressed without using other substances. Pellets of morphine were implanted aseptically in the back of the animals. As it will be reported under "Results", the pellets take some days to be absorbed. Knowing this data, as well as the weight of the pellet and the animal weight, it is easy to estimate the daily absorbed dose of morphine by kilogram of mouse.

Nalorphine (Allyl-normophine) hydrobromide in water solution was injected in the anterior wall of the chest, at the dose of 10 mg per kilogram of weight.

Results

A) Rate of absorption of morphine administered as pellet. — Subcutaneous tissue accepts very well the pellet of morphine. In a great percentage of cases it is surrounded by a thin capsule, well irrigated and occasionally bloody (in some opportunities the capsule may be thick); sometimes, the capsule also contains a greenish amorphous substance like pus, very easily separable; in other cases, a serohemorrhagic liquid is found. However, absorption is regular in spite of anatomical variations (Fig. 1).

Results expressed in figure 1 (each point corresponds to one observation) indicate that the pellet disappears approximately between 30 and 32 days after the implantation.

B) Effects of morphine. — In order to avoid errors it was necessary to train the mice, especially in those cases dealing with pain threshold, muscular tension and the "complex test". The daily exercise of animals in similar conditions could provoke inespecific responses. Because of this, it was necessary to perform a large number of blanks and to discard many animals. When the mice were considered to be well adapted to start the experiments, the observation began; thus, 19 animals were studied during 54 days without considering the training period. The first 24 days were taken as control, and then, the first implantation was performed, giving a daily absorption per kilogram of 66 mg of morphine. The second implantation was done 14 days after the first one, obtaining a daily absorption of morphine per kilogram of 311.3 mg. The third implantation (27 days after the first one), gave an absorption of morphine by kilogram and by day of 1070.5 mg.

Results showed that the animals have variable reactions (Table I) and that the same mouse can modify its performance from one day to another (Table II). For this reason, it was not possible to analize statistically the results. It was thought wiser to study each animal in particular and to verify how morphine changes the values obtained before the implantations. In this way, Table I was elaborated; the following facts may be infered from it:

a) The general attitude of the mice is modified: the animals become calm, depressed or catatonic and occasionally excited, with hypermotility; frequently they become aggressive. The effect of the second implantation is something different: the drug is not so depressive, and in some animals it has no action. Results of the third implantation contrast especially with the first one, because it does not modify the attitude of the mice.

b) The drug produces analgesia. When morphine is firstly implanted it is very rare to find any animal with similar reactions to the control. On the contrary, after the third implantation, in some occasions one observes that the threshold is increased. The second pellet induces intermediate effects between those already referred.

TABLE I

Action of morphine implantations on the attitude, Straub phenomenon, exophthalmus, analgesia, muscular tension and the so called "complex test"

MOUSE Nº	ATTITUDE			STRAUB PH.			EXOPHTHAL MUS			ANALGESIA (1)			MUSC. TENS. (2)			COMPLEX TEST (3)								
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st			2nd			3rd		
1.1	cd/			-/-			o	o	-/-	i	i	i	?			d	i	i						
1.2	cd/	ct	o	-/-	-/-	-/-	o	o	-/-	i	i	i	?	?	?	i	d	i	?			?		
1.3	cd/	ct		-/-	-/-	-/-		-/-		i	i		d	?		i	i	i	d	i	i			
1.6	cd/	ct		-/-	-/-		-/-	-/-	-/-	o	o		d	?		i	d	d	o			i	o	o
2.2	ct	ce	o	-/-	-/-	-/-	-/-	-/-	o	i	?	?	d	o	d	?	d	i	i	o	i	i	o	o
2.3	ce	o	o	-/-	-/-	-/-	o	o	-/-	i	?	o	d	d	o	o	o	i	o	i	i	o	o	o
2.5	c	o	o	-/-	o	-/-	o	o	o	i	?	o	d	d	o	o	o	o	?			?	o	o
2.6	c	t	o	-/-	o	-/-	-/-	-/-	o	i	o	o	d	o	i	o	o	i	o	o	i	o	o	o
4.2	e	ct	o	-/-	-/-	-/-	o	-/-	o	i	o	o	d	o	?	?	o	d				o	o	o
4.3	c	o		-/-	-/-	-/-	-/-		-/-	i	i	i	o	i	o	d	o	d	i	o	i	o	i	o
4.4	ct	o	o	-/-	o	o	o	o	o	i	o	o	o	i	i	d	o	i	o	o	o	o	o	o
4.5	ce	t	t	-/-	-/-	o	o			d	i	i	o	o	i	o	o	o	o	o	o	o	o	o
4.6	cd/			o			o	o	o	i			o	d	o	i	i	i						
4.7	cd/	o	o	o	o	o	-/-	o	o	o	o	i	i	o	o	o	o	o	i	i	i	o	o	o
5.1	ct	o	o	-/-	-/-	o	-/-	-/-	o	i	i	o	o	o	o	i	o	o	o	i	i	o	o	o
5.2	ct	o	o	-/-	-/-	-/-	o	-/-	o	i	i	o	o	o	o	o	o	o	o	o	o	o	o	o
5.3	c	o	o	o	o	-/-	o	o	o	i	i	o	i	o	o	i	o	i	i	i	o	o	o	o
5.4	c	o	o	-/-	-/-	-/-	-/-	-/-	o	o	i	i	o			o	o	o	o	o	o	o	o	i
5.5	c	o	o	-/-	-/-	-/-				i	o	o	i	o	i	i	o	o	i	o	o	o	o	o

c: catatony - d/: depression - t: tranquility - e: excitation - o: without change - -/-: positive result - i: increase - d: decrease - ?: dubious interpretation - 1st, 2nd, 3rd: observations after the first, second and third implantation. (1) valued by the time taken by the animal to raise the posterior limb when it is put on a metallic plate heated to 56°; (2) valued by the time taken by the animal to loose a metallic gauze of 45 gm of weight, when it is suspended by the tail; (3) valued by the time taken by the animal to leave the first surface, to cross the cylinder and to leave the second surface.

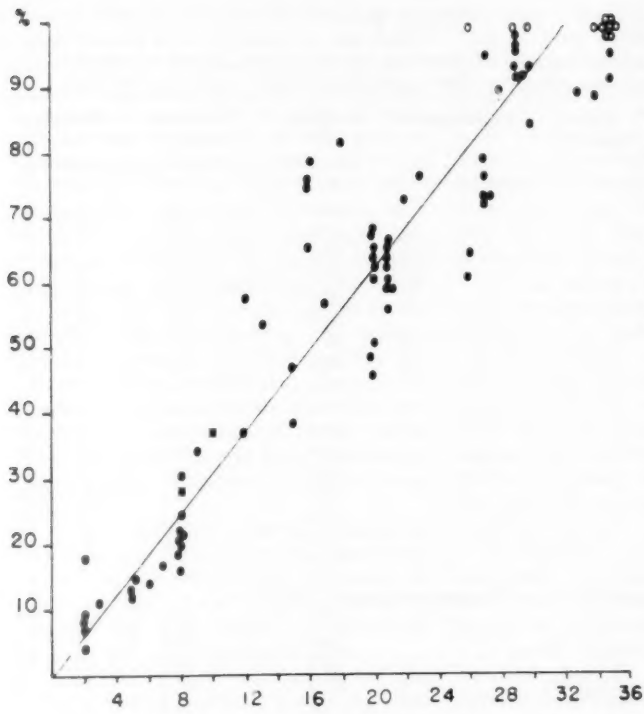

FIG. 1. — *Absorption rate of morphine pellets implanted in the subcutaneous tissue of the back in mice. Ordinate: percentage of absorption. Abscissa: time in days. Each point represents one observation; the white circles indicate that the pellets had disappeared because of complete absorption.*

c) Straub reaction (rigidity and erection of the tail) is very frequently observed and has a great intensity after the first implantation. However, its frequency tends to decrease with the new ones; thus after the third, there is a large number of mice in which the Straub phenomenon persists. As several authors indicate ([1]), the Straub erection is accompanied by lordosis, extension, paralysis of the hindlimbs and prominence of the perineum; these signs have the same evolution that has the Straub phenomenon.

d) Exophthalmus has been described as an interesting effect of morphine. In our observations, the exophthalmus was not very frequent, perhaps due to the plain visual appreciation. As the implant of morphine was repeated, the frequency of exophthalmus does not change appreciably.

e) During the first days after implantation the animals loose weight or the ponderal curve stops; some die during the first three days. Afterwards, mice normalize day by day.

f) In one opportunity the action of morphine was studied in rats. It was observed that a great number of them frequently fell down from the table. In that occasion it was thought that some alteration in equilibrium or in muscular tension could be present. In order to inquire these perturbations in mice, they were obliged to pass through a cylinder of wood and the muscular tension was tested. The animals crossed the wooden bridge without difficulty; therefore, it could be de-

TABLE II

Effect of 3 consecutive morphine implantations on the attitude, Straub phenomenon, exophthalmus, analgesia, muscular tension and the "complex test" in one mouse

Day	Attidude	Straub phen. (1)	Exophthalmus	Analgesia (2) (seconds)	Musc. tens. (3) (seconds)	Complex test (4) (seconds)
1				10.5	179.0	5 3 5
4				37.0	17.5	13 5 3
5				31.0	10.0	
6				21.0	22.5	6 4 5
8				26.0	8.0	22 4 5
9	t			28.0	8.0	13 3 4
11				36.0	7.5	12 3 3
12				20.5	23.0	8 4 3
15	t			95.0	8.0	
16				18.0	13.0	9 4 7
17				29.0	20.0	6 3 8
18				30.0	19.0	14 4 9
19				14.5	18.5	3 4 3
20				22.0	21.0	105 7 158
22				47.0	14.5	16 5 10
23				31.0	75.0	20 4 6
24				32.0		
24	79 mg of morphine (base) per kilogram weight and per day					
25	ct	-/- 45°	—	20.0	103.0	61 7 160
26	ct	-/- 45° (P)	-/-	26.0	162.0	10 3 70
29		—	—	45.0	148.0	14 3 10
31	ct	-/- 45° (P)	—	17.0	> 180.0	11 8 15
33	e	—	—	8.0	145.0	21 5 9
36				21.0	130.0	17 5 7
37	t	—	—	28.0	> 180.0	
38				13.5	> 180.0	4 5 11
38	298 mg of morphine (base) per kilogram weight and per day					
39	t	-/- 45° (P)	—	46.5	138.0	10 5 25
40	ct	—	—	34.0	140.0	4 4 22
41			—	28.0	> 180.0	2 6 64
43		—	—	62.0	173.0	5 5 12
46	ct	-/- 20° (P)	—	76.0	> 180.0	11 3 6
48			—	100.0	160.0	5 4 8
50	ct	—	—	35.5		13 7 6
51	939.5 mg of morphine (base) per kilogram weight and per day					
52	ct	-/- 20° (P)	—	123.0	178.0	9 6 20
53		—	—	30.5	> 180.0	6 4 5
54	t	-/- 30°	—	79.0		

t: tranquility - c: catatony - e: excitation - -/-: positive result - —: negative result - (P): the sign appears when it is provoked. (1) The number indicates the appreciation in degrees of the tail elevation. (2) Time taken by the animal to raise the posterior extremity when it is put on a hot metallic plate (56°). (3) Time taken by the animal to loose a wire gauze of 45 gm of weight when it is suspended by the tail. (4) Time taken by the animal to abandon the first surface, to cross the bridge and to abandon the second surface.

duced that no important alteration of equilibrium was produced by the drug. Nevertheless, in some animals the time taken to pass through the cylinder was prolonged. In order to study if morphine was responsible of this prolongation through a mechanism that could indirectly alter the equilibrium other difficulties were added to the test, and thus the "complex test" was born (see Method). Whenever the animal delayed in crossing the cylinder, the time taken to leave the first surface and to arrive to the cage after passing the bridge, were prolonged too. For details, see Table I. The modification of the mice performance induced by the three implants are also indicated in the table. It is of interest to add that, practically, no mistake was done in performing the test.

g) The animals receiving morphine develop a tolerance to the anesthetic action of the ethyl ether, measured by the quantity of the drug necessary to induce the same degree of anesthesia during the same period of time.

Ten to 14 days afterwards all the effects studied vanished, appearing after a new dose of morphine; normalization is obtained sooner after the second implantation, and it is still faster, when a third pellet provokes alterations. Thus, one may accept that the morphine develops tolerance in the mouse.

C) Action of nalorphine: the abstinence syndrome. — Though a great number of mice has been employed to study the abstinence syndrome, only those described in section B will be analized in this opportunity. The animals have received the doses of morphine as it was indicated (see page 70). Nalorphine hydrobromide in doses of 10 mg per kilogram of weight (refered to nalorphine base) was injected 28 days after the first implantation.

Two or 3 minutes after the administration of nalorphine, the following symptoms appear when the animals are placed on the observation table:

a) A very intense increase of the spontaneous activity is observed. The animals become restless, and not only walk more and more, but run disorderly and without an apparent object. This hypermotility is very peculiar because the mice drag the perineal zone over the table. Hypermotility (Fig. 2) is very frequent and practically is observed in all the animals.

b) The animals jump repeatedly, almost in stereotipied form, either in the middle of the table, getting or not the top of an object left on it, or looking for the edge of the table in order to throw themselves to the ground. If the mice are put again on the table, they repeat the movements many times. Before jumping they sit or stand on the posterior extremities and begin to smell in diverse directions.

To facilitate the nominations of the symptoms indicated in paragraphs a) and b) they will be designated as the period of hypermotility.

c) It is possible to observe small tremors that in some opportunities become convulsions. When the animal is suspended by the tail, tremors increase in number and in intensity; irregular movements and brief and small convulsions appear. These events will be designated as "tail reaction". It is very frequent and it can be found in almost all animals. In some mice it is possible to observe generalized twitches and in others, tonic contractions of the extremities and of the back that can be localized and produce asymmetric positions. In others there is hyperreflexia and an increased irritability.

In order to save space all the symptoms described in c) will be refered to as neurological phenomena.

d) The frequency and intensity of the respiration increases practically in all the mice.

e) The tear secretion is increased;

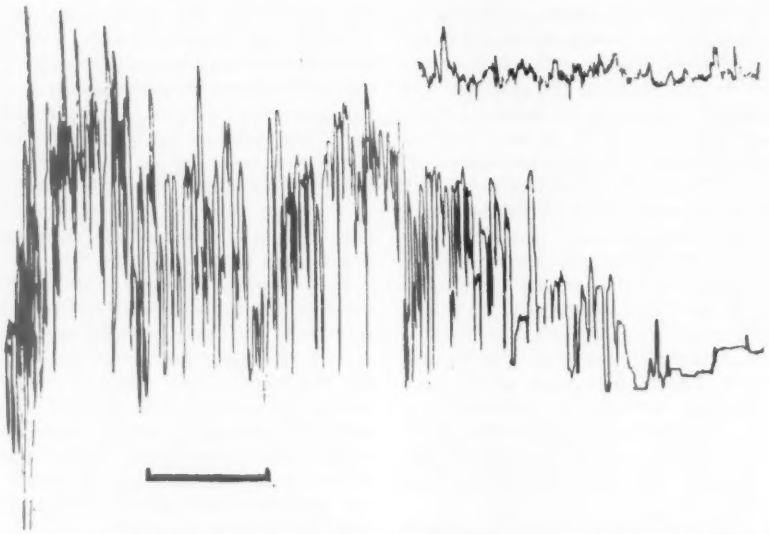

FIG. 2. — *Hypermotility in the nalorphine induced abstinence syndrome. Right and upper angle: control. Below, record of hypermotility in the abstinence syndrome immediately after the injection of nalorphine. Time: five minutes.*

thus, the movements of the eyelid are difficulted and the mouse stays with the eyes closed or semiclosed. It is frequent to observe an increased number of depositions with soft or liquid feces. It is not rare to observe mictions during the minutes following the injection of nalorphine. It is possible to find priapism and it would not be strange that some ejaculations may be produced because of the observed rhythmic movements of the pelvis.

f) All the signs described appear very soon after the injection of nalorphine and last approximately for half an hour. They vanish in intensity and frequency, reaching a period of profound immobility which doubles the duration of the already mentioned symptomatology.

If nalorphine is administred to normal animals it does not induce any of the mentioned perturbations; the mice become quiet and drowsiness appears by the action of 10 mg per kilogram of weight.

When one studies the symptoms of nalorphine in the morphinized mice it is possible to learn that the manifestations are similar to those described in other animals and in men as "abstinence syndrome" (4).

The abstinence syndrome is seldom observed with all its symptoms in one mouse. On the contrary, there frequently appear only some ones; the hypermotility, the tail reaction, the dyspnea, the diarrhea and the immobility are the most characteristic and constant signs. The intensity changes according to the mouse. Occasionally the convulsions are so violent that the animal dies a few minutes after the administration of nalorphine. It was thought possible that the most intense syndromes were due to intravenous administration of nalorphine, because of an hemorrhagic zone found at the in-

jection place. But this was not the case; when nalorphine was given into the vein, the abstinence syndrome appeared sooner with approximately the same intensity.

The indicated description of the abstinence syndrome belongs to that obtained with our mice. Nevertheless, the symptoms may change according to the strains. Thus, in the mouse DBA (deep brown) from Dr. Hoeckel's stock (*) the period of hypermotility is weak and short; on the contrary, the immobility is so profound that the animals rest wherever they are placed as if they were lethargic.

Discussion

The classic effects of morphine have been confirmed in mice when the drug is administered as pellets: changes in attitude, presence of Straub reaction, exophthalmus, analgesia, lost of weight, resistance to analgesic properties of ethyl ether, etc.

Tolerance to morphine is developed little by little. It the dose of the drug is large enough and does not kill the animal in the first 2 or 3 days, the mouse will accept afterwards very high quantities in short time. The animals here employed absorb more than 1 gm per day and per kilogram 27 days after the first implantation. In other occasions, not described in this paper, animals absorbed more than 2 gm 20 days after the first administration. On the other hand, one can administer at once an amount of morphine that represents 600 mg of daily absorption per kilogram of weight, without killing the mouse.

As it was referred, pain was studied placing the animal on a metallic plate heated to 56°. Greater temperatures induce burns, as also has been indicated by others (6). When the test is performed daily during a long period they learn to recognize the heated plate and rapidly do the characteristic sign, at which time the test is ended; other, sitting on the posterior limbs do not put the hand in the mouth. A third group jumps down from the plate; in a fourth, the sign was made so rapidly, that it was not possible to assure if it was or not conditioned by heat. Responses to the thermal stimulus seems more regular if one measures the time taken by the animal to carry the posterior limb to the mouth. Because of this, in our experiments we considered this fact as the characteristic sign. It is possible that the Woolfe's method may be adequate for studying the analgesic properties in only few opportunities in the same animal, but when the test must be repeated during a long time, day by day, it has less value for the discussed reasons. This may be the cause of variabilities in the analgesic threshold shown in Table II.

Muscular tension was verified by the time elapsed before the mouse looses the wire gauze of 45 gm of weight when the animal is suspended by the tail. Obviously the test implies other factors that may induce mistakes: abnormal position, movements performed by the animal on the wire gauze, etc. Perhaps these will be the reasons why the muscular tension appears to be decreased by the influence of morphine, result that does not agree with the current observation that is is more difficult to separate the animal from the fingers when it is handled. It could be objected that other factors besides muscular tension would dissappear by training. Nevertheless, it is necessary to remember that the experiment started after the animals were used to it.

Morphine does not induce an important perturbation of equilibrium. The only abnormal sign occasionally presented is a prolonged time in crossing the wooden cylinder. When it is

(*) We thank Dr. Hoecker for the mice supplied.

present, the time taken to leave the first surface and to reach the cage after crossing the bridge, are also prolonged. One explanation of the phenomenon may be found in the immobility and the catatonic status which may produce the drug.

The abstinence syndrome in the mice is rich in manifestations. Unfortunately, it is difficult to evaluate its intensity in order to establish comparisons. Symptoms vary in quantity, intensity and frequency. In some mice the period of hypermotility persists for a long time; in others, it is short, and finally, when the period of immobility appears, it is difficult to evaluate its intensity and duration. Thus, comparisons must be rough and based on subjective appreciations; these facts agree with those related by Carter and Wikler (2).

Seevers (9, 10) has emphasized the exigence of a correct method in order to induce physical dependence in small animals. Furthermore, he has postulated that the administration of doses of morphine, separated by large intervals of time is an experimental error. In this paper it is demostrated that morphine implanted as a pellet may induce a very intense abstinence syndrome rich in signs after a relatively short time Hanna (5) obtains in rats, after 20 days of treatment, an abstinence syndrome neither so intense nor with so many symptoms as the one reported in the mouse in this paper. He injects morphine every 4 hours, arriving to the very large dose of 2.2 gm a day. The difference must be due to the dissimilar species employed, or perhaps, to the continuous administration of morphine which produces the implantation of the pellet.

Summary

1) Pellets of morphine have been implanted in the subcutaneous tissue of mice. The pellets are well tolerated locally; its absorption is regular and is completed during 30-32 days.

2) Morphine administered as a pellet provokes its classic effects. Furthermore, the equilibrium, muscular tension and a "complex test" are not modified.

3) Morphine as a pellet induces tolerance, phenomenon that progresses gradually. In the experimental conditions here reported all signs studied vanished at least in 13 days. On the other hand, when the compound is administered as a pellet mice tolerate large doses of morphine.

4) When the action of morphine is interrupted by the effect of nalorphine, the abstinence syndrome appears; it has many symptoms and they are very intense. Unfortunately, symptoms are so varied that it is not possible to standardize the syndrome in order to establish comparisons.

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VASCULAR CHANGES IN THE RETINA OF DIABETIC RATS

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MANY different studies have been done on the retina of the rat in an attempt to obtain a satisfactory method of clearly presenting the arteries, veins and capillaries so that their possible changes in experimental diabetes can be studied.

The first tests were made with intra-arterial injections of India ink in anesthetized animals. This technique was not successful because only the vessels' borders could be seen, and not very clearly.

By a technique using silver nitrate intravascularly. Hausler and Sibay (1959) achieved results which induced us to make studies using silver ammoniacal carbonate of Río Hortega, also administered intravascularly. By this method sufficiently clear images were obtained in normal animals (Musacchio and Palermo, 1959). Cairns, in the same year, described the vascular distribution in the retina of the normal rat, based on the work of Michaelson (1954), who completed and enlarged the work by Hesse (1880). Sudak et al. (1958) observed vascular distensions in the venous walls of cheek pouchs of hamsters having alloxan diabetes for 90-120 days.

The present work shows that alloxan diabetes produces marked changes in the arteries and veins of the retina in rats.

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MATERIALS AND METHODS

The animals used were forty adult rats, raised in our Institute, with moderate to severe alloxan diabetes. During the entire experiment they received forced feeding and the degree of their diabetes was controlled weekly through determinations of urine glucose and monthly by determinations of glycemia. The control rats were forced fed and were injected with alloxan but did not become diabetic.

The technique used for the observation of the retina was the following: under general anesthesia by ether, the inferior vena cava and the abdominal aorta were ligated. The abdominal aorta was catheterized with a polyethylene tube up to the ventricle and the jugular veins were sectioned to establish an open circuit.

Then through the aorta and at

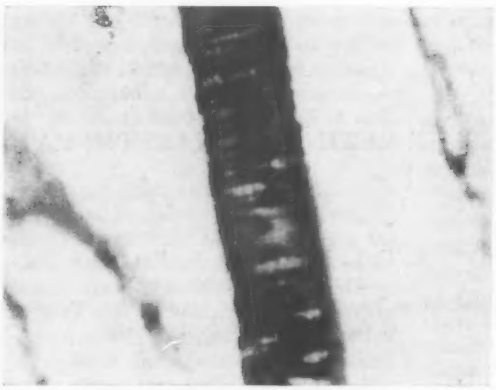

FIG. 1. — *Normal artery in a control rat (weight: 120 grams). The diameter is uniform. The transverse striae are muscular fibers (x 480).*

approximately 100 mm Hg pressure, 1 and 10 % formaldehyde as well as subsequently a strong solution of silver carbonate (*) were injected. The eyes were removed and placed for 15 minutes in 1 % formaldehyde at 35-40° C; they were washed with distilled water and an incision was made in the upper temporal quadrant of each eye. They were then placed again in 1 % formaldehyde for 1 to 5 hours to complete the fixation.

Dissection and smear of the retina: the eyes were marked for orientation in the upper limbic zone, from which point the corneas were removed with scissors and the lens extracted. An incision was made in the outer upper quadrant, including all the membranes, to determine the orientation of the previously smeared retina. The retina was separated from the choroids with scissors to the junction of the optic

(*) Strong solution of silver carbonate:

10 %	silver nitrate	100 ml
10 „	sodium carbonate	300 „
	bidistilled water	300 „
add 10 „	silver nitrate	100 „

A yellowish-white precipitate forms which clears when ammonia is added drop by drop and shaken.

Important: do not add ammonia to excess.

FIG. 2. — *Normal vein in a control rat (weight: 53 grams). The diameter is uniform. Note the endothelial borders and nuclei (x 480).*

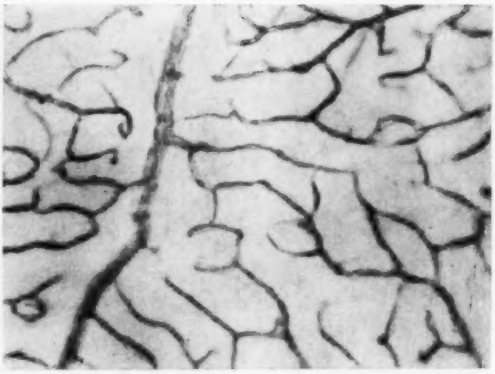

FIG. 3. — *Superficial capillary net in the retina of a normal rat (weight: 110 grams). One vein with direct A. V. anastomosis, with a free perivenous area and the beginning of deep capillaries are shown (x 120).*

nerve. The optic nerve was sectioned after the choroids and sclera had been placed to one side and the retina to the other. The retina was then passed through 1 % sodium hyposulfite and desiccated by the usual technique for silver dyeing. The retina was mounted on a slide and several radial incisions made.

RESULTS

Figures 1 and 2 show the normal appearance of the arteries and veins. It can be seen that this technique shows the structure of the cells and fibers of the vessels. Figure 3 shows the normal superficial capillary net and the beginning of the deep capilaries on different levels.

The pictures of the diabetic rats (Figs. 4, 5, 6 and 7) show changes, the predominant one being a vascular dilatation with the following characteristics: 1) it is seen initially in the venous net and later becomes generalized and uniform in its distribution, remaining constant during the entire period from the sixth to the sixteenth month of diabetes; 2) the appearance of papillary vessels or vascular stars is accompanied by a dilatation of the

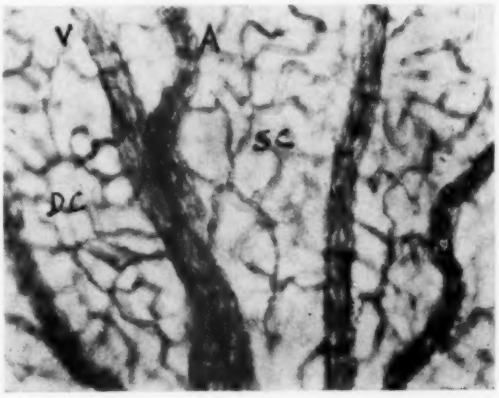

FIG. 4. — *Rat with severe diabetes of 16 months duration (weight: 271 grams) showing three arteries and two veins. The arteries are flexuous with few muscular fibers; an arterio (A)-venous (V) crossing is seen, which is abnormal at that level, lacking muscular fibers. The vein adapts itself to the flexuosity of the artery. Note the flexuous capillaries of the superficial net (S C), as well as those of the deep capillary net (D C) (cf. Fig. 3), due to the atrophy of the retina (x 120).*

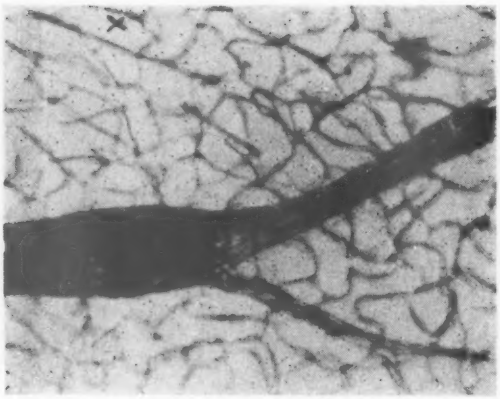

FIG. 5. — *Rat with moderate diabetes of 16 months duration (weight: 270 grams). Artery: proximal end very dilatated with loss of muscular fibers, a narrow thinned wall shows the transverse capillaries. Capillaries: arterial in the superficial net very flexuous and interwoven with the veins in the deep net, which are dilated (x 80).*

merging area whose surface is also increased (Fig. 7); 3) the most striking finding is arterial dilatation. The vessels reach a large caliber in some cases which is fusiform in others. The arterial dilatation ends abruptly and returns later to a normal caliber (Fig. 5). The vessels are elongated and therefore flexuous (Fig. 6). Another point to consider is the lessening of the thickness of the wall at the expense of the disappearance of the muscular fibers in the dilatated zones; 4) the capillary net: the superficial and the deep capillary nets are found on the same level, due possibly to a thinning of the retina, which is not seen in the normal retina (Fig. 4). The deep net, which is formed entirely by venous capillaries, is also dilatated and flexuous.

These changes were bilateral in all the cases and observed in 39 out of the 40 rats studied, but were more marked in the animals with severe diabetes.

DISCUSSION

The technique described allows a clear observation of the arteries and

FIG. 6. — *Rat with severe diabetes of 15 months duration (weight: 235 grams). Artery and capillaries are flexuous and dilatated with a loss of transverse fibers. Note twisting of all the axis (x 320).*

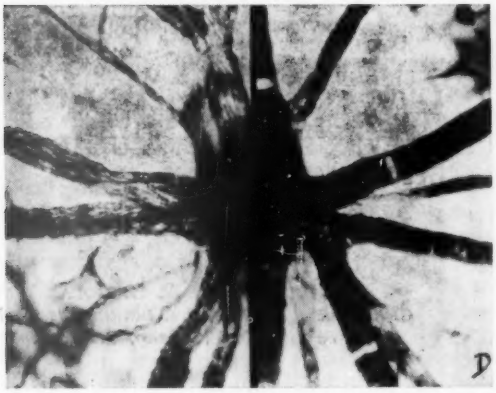

FIG. 7. — *Changed picture of vascular emergency, right side (D) in a normal rat (weight: 290 grams) and a rat with severe diabetes of 16 months duration (weight: 295 grams); left side (I) (x 80).*

veins and the capillary net of the retina. These observations have been extended to other organs and will permit us to make similar studies.

In the rat with moderate or severe alloxan diabetes of 6 to 16 months duration the changes are reduced predominantly to vascular dilatations and, in these experiments, do not show the polymorphic changes seen in human diabetic retinopathy.

Studies in rats with diabetes after subtotal pancreatectomy are in progress.

SUMMARY

White rats from our Institute strain with severe or moderate alloxan diabetes were used through out the experiment; 40 of these animals were forced fed during 16 months with weekly determination of glucose in urine and glycemia measured once a month. Alloxan injected non-diabetic rats were used as controls.

Under ether anesthesia and after ligature of the vena cava and abdominal aorta, they were injected, in the thoracic aorta and the ventricle, with silver carbonate. Afterwards the eyes were removed, fixated, the retina dissected and studied under the microscope.

The most characteristic changes were dilatation of the vessels, specially the arteries and loss of the muscular fibers, without hemorrhages.

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LA ACOMODACIÓN DEL MÚSCULO VENTRICULAR DE TORTUGA (*)

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EL fenómeno de acomodación, modificaciones de los umbrales de estimulación por el paso de corriente directa en un tejido excitable, ha sido analizado por Solandt (1936), Bernard (1942) y por Rosenblueth y del Pozo (1942) en el nervio y en las motoneuronas por Araki y Otani (1959). El propósito de este trabajo fué el de estudiar el comportamiento del músculo cardíaco en lo referente al proceso de acomodación y los cambios electrotónicos de su excitabilidad.

MÉTODO

Se emplearon corazones de la tortuga *Kinosternon hirtipes*. Dos tipos de preparaciones fueron usadas: ventrículo aislado y ventrículo perfundido. El ventrículo aislado fué fijado a una lámina de corcho cubierta con papel de filtro humedecido en solución salina y el ventrículo perfundido lo fué de acuerdo a la técnica de Straub-Führer. Ambas preparaciones se colocaron en una cámara húmeda. Por medio de secciones (en el ventrículo aislado) y de ligaduras en la base del ventrículo en el perfundido, se abolió totalmente el automatismo. Para humidificar y perfundir el ventrículo se utilizó líquido de Tyrode, ajustando su pH a 7.2 con $NaH_2PO_4 + 2H_2O$. Como electrodos de estímulo se utilizaron hemicélulas de calomel saturado de 3 cm de diámetro. El contacto con la preparación se hizo a través de un puente de agar-Ringer que terminaba en un hilo embebido en la misma solución. Estos electrodos se comportaron como impolarizables con los voltajes empleados (5 a 70 voltios de corriente directa). La impolarizabilidad de los electrodos se comprobó de las dos siguientes maneras: 1) en un óhmetro conectado en serie con los electrodos, sumergidos en solución salina se leyó la corriente correspondiente a los diferentes voltajes aplicados; 2) se hicieron pasar corrientes de diversos voltajes a los electrodos de estímulo en serie con un osciloscopio. Al mantenerse constante la rela-

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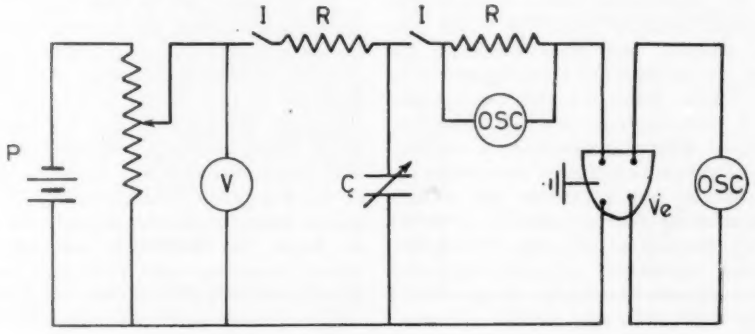
ción entre voltaje y resistencia en el primer caso y al obtenerse un desplazamiento instantáneo y fijo del rayo del osciloscopio, en el segundo, se concluyó que no se desarrollaba fuerza electromotriz de polarización en los electrodos.

Para la determinación de la acomodación se utilizaron corrientes exponencialmente crecientes. Un circuito combinaba adecuadamente condensadores en paralelo y resistencias en serie con el ventrículo de acuerdo al esquema de la figura 1 (Solandt, 1936). Para las curvas de intensidad-duración se agregó a este circuito una llave inversora. La resistencia del circuito era de 120.000 ohms, la de los electrodos 40.000 ohms y la de la preparación 4.000 ohms. En el transcurso de los experimentos esta última no tuvo cambios mayores de 1.000 ohms. Las constantes de tiempo obtenidas con los condensadores utilizados (1-11 μf) fueron hasta de un segundo. Para los cambios electrotónicos se utilizaron dos estimuladores (Grass, modelo S4B) acoplados en paralelo. Los estímulos se pasaron a través de unidades que los separaban de tierra. Para el registro se utilizaron electrodos de plata conectados por puentes de agar-Ringer terminados en una mecha de hilo apoyada sobre el ventrículo. Los registros, tanto de la respuesta ventricular como de los potenciales aplicados, fueron fotografiados en la pantalla de un osciloscopio de rayos catódicos de doble rayo, utilizando su amplificador acoplado directamente. Para el registro de las respuestas repetitivas se usó un galvanómetro de cuerda.

Para medir la excitabilidad eléctrica del corazón se construyeron curvas de intensidad-duración, con descargas de condensadores, antes y después de cada medida de la acomodación. Los experimentos en donde las curvas iniciales y finales tuvieron diferencias significativas fueron desechados. Ambos tipos de preparaciones mantenían iguales condiciones de excitabilidad durante dos horas; de ahí que en la mayoría de las determinaciones se usara la preparación de ventrículo aislado, por ser de técnica más fácil. Los experimentos fueron realizados a una temperatura que varió entre 18° y 23°C.

La determinación de las curvas de acomodación y de los cambios electrotónicos, se hicieron pasado un lapso de 30 min de colocado el músculo ventricular en la cámara húmeda, pues durante este tiempo se observaron os-

FIG. 1. — *Diagrama del circuito eléctrico utilizado para estimular con corrientes exponencialmente crecientes. P: pila seca 45-90 volts. V: voltímetro. C: condensadores variables. R: 200,000 Ω. I: llave interruptora. OSC: osciloscopio. V_e: músculo ventricular.*



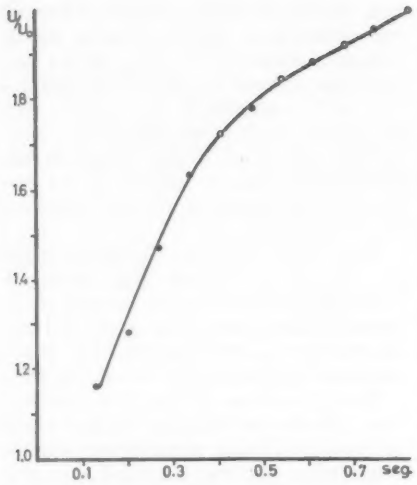


FIG. 2. — *Curva de acomodación determinada con corrientes exponenciales. Abscisas: constante de tiempo de carga de los condensadores. Ordenadas: cociente entre voltaje máximo aplicado y voltaje reobase.*

cilaciones de la reobase y de la resistencia total de la preparación.

RESULTADOS

Aplicación de corrientes exponencialmente crecientes. — Se hicieron determinaciones del voltaje mínimo necesario para provocar una respuesta ventricular (U), utilizando pulsos de corriente exponencialmente creciente, y del voltaje umbral, utilizando corriente directa de crecimiento instantáneo (U_o). Este último valor se midió varias veces en el transcurso de los experimentos. Una vez determinado el valor umbral (U_o) se procedía a buscar una respuesta ventricular con estímulos que tenían constantes de tiempo variables (100-900 mseg); después se procedía de manera inversa, aplicando primero estímulos con constantes de tiempo largas, hasta llegar a estímulo con constantes cortas. Con cada constante de tiempo se investigó el mínimo voltaje que producía una respuesta ventricular.

La curva de la figura 2 fué obtenida graficando en las abscisas constantes de tiempo y en las ordenadas el voltaje máximo de los pulsos exponenciales U_o sobre el voltaje umbral. Al disminuir la derivada de los pulsos, se observó que se necesitaban mayores voltajes para alcanzar el umbral. La mitad de las curvas obtenidas tuvieron una configuración paraboloide (Fig. 2), pero esta definición matemática no pudo ser aplicada a la totalidad de las mismas. Las curvas presentaron una disminución en la pendiente cuando se estimuló con corrientes exponenciales de larga duración.

Influencia de los pulsos largos subreobásicos catódicos y anódicos sobre la excitabilidad. — Se aplicaron pulsos largos de 0.5 a 1.5 seg de duración con una intensidad que variaba entre el 45 y 90 por ciento de la reobase, la cual fué determinada por un pulso de corriente directa catódico de duración larga. Por los mismos pares de electrodos impolarizables se enviaron pulsos catódicos de prueba de 1 mseg de duración para determinar umbrales. Los intervalos de aplicación de estos pulsos medidos a partir de la iniciación del pulso largo, eran variables. Se exploraron también los umbrales después del fin del pulso largo condicionante.

En la figura 3-I se observan los resultados obtenidos en un experimento donde se aplicó un pulso condicionante catódico de 80 por ciento de la reobase. Durante la fase inicial se vió una disminución del umbral, que fué aumentando hasta sobrepasar el valor de control, manteniéndose este aumento hasta el fin del pulso condicionante. Al explorar inmediatamente después del pulso largo, los umbrales tuvieron un brusco aumento para disminuir exponencialmente hasta los valores iniciales.

Con pulsos anódicos largos, y estí-

mulos de prueba catódicos de 1 mseg de duración (Fig. 3-II), aparecieron modificaciones opuestas a las descritas para los pulsos catódicos. El umbral se encontró inicialmente aumentado, para disminuir después y quedar por debajo del valor inicial, hasta el cese de la corriente. Los cambios que se observaron después del cese de la corriente anódica fueron opuestos a los determinados por la suspensión del pulso catódico.

Los cambios precedentemente descritos están en relación directa con la intensidad del pulso aplicado como lo muestra la curva de la figura 4, la cual fué determinada en la siguiente forma. Se eligió un intervalo en donde se observó acomodación; se varió la intensidad del pulso condicionante y para cada intensidad se probó el umbral. La acomodación apareció con voltajes mayores del 45 por ciento de la reobase y a medida que esta intensidad se iba aumentando, iba creciendo el proceso de acomodación. El aumento del voltaje del pulso polarizante, provocó modificaciones del momento de aparición de la acomodación, por lo que no se pudo dar valores de tiempo precisos, para definir este proceso.

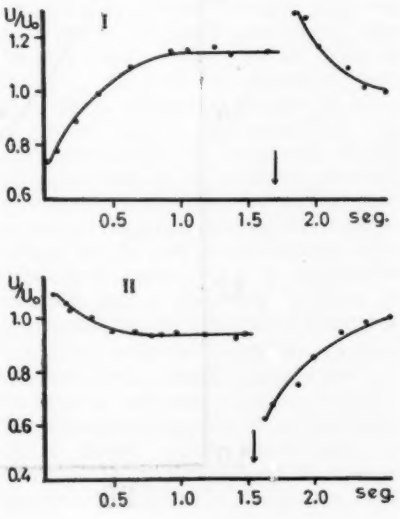
Respuestas repetitivas. — La aplicación de pulsos largos (40-100 seg) de corriente directa, 2-3 veces umbral, provocó la aparición de respuestas repetitivas en el ventrículo, durante los primeros 10 seg de iniciada la aplicación del pulso. El mismo procedimiento se repitió en el corazón de tortuga "in situ" con electrodos impolarizables conectados al ventrículo y asegurándose que todas las respuestas tuvieran origen ventricular. En este caso las respuestas se obtuvieron durante todo el tiempo de aplicación del pulso.

Discusión

La necesidad de usar ventrículos desprovistos de automatismo, está determinada por la duración de las corrientes empleadas necesarias para la construcción de las curvas que duraban más que el ciclo de recuperación de la excitabilidad ventricular.

Medida de la acomodación por la aplicación de corrientes exponencialmente crecientes. — Con el circuito utilizado se obtuvieron corrientes que crecen exponencialmente con una constante de tiempo que está determinada por la capacidad. Con estas corrientes de crecimiento lento el voltaje para alcanzar el umbral aumentaba gradualmente. Al graficar en coordenadas

Fig. 3. — *Cambios de umbrales en las respuestas que se desarrollan durante o después de la aplicación de pulsos condicionantes. La amplitud del pulso de prueba fué medida a partir del cero del pulso largo. Abscisas: tiempo. La flecha indica el fin de la aplicación de los pulsos largos condicionantes; el principio de éstos está determinado por el origen. Ordenada: cociente entre el voltaje necesario para alcanzar el umbral (U) y el umbral obtenido con un pulso catódico de 1 mseg de duración (U_o) (U/U_o). En las ordenadas el valor 1.0 representa el umbral.*

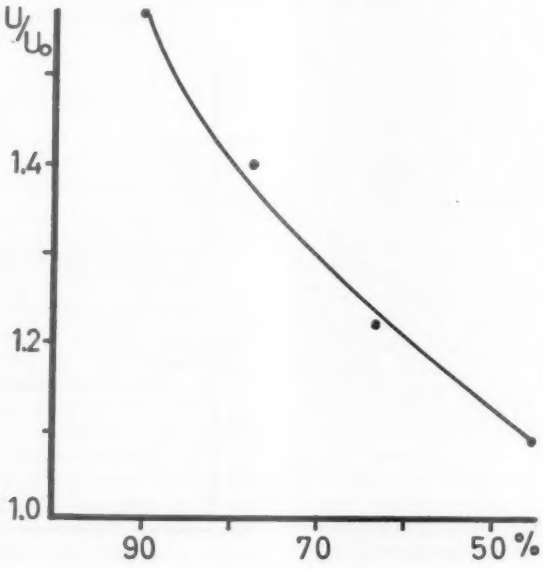


cartesianas, la constante de tiempo de carga de los condensadores contra el voltaje necesario para estimular, se obtienen curvas que son demostrativas de la acomodación (Hill, 1936). En nuestras curvas se graficó la intensidad máxima para que con una constante de tiempo dada, se alcanzara el umbral. Esta intensidad máxima no es a la que la respuesta ocurrió, pues experimentalmente se observó que dichas respuestas se producían al llegar al 70 u 80 por ciento del voltaje medido.

A los resultados observados, se les aplicaron las ecuaciones que se han desarrollado para nervios. Las propiedades eléctricas cualitativamente semejantes entre los tejidos nerviosos y el músculo cardíaco, permiten esta comparación. Quedó descartada la aplicación de la ecuación de Hill (1935) que predice una relación lineal entre el umbral relativo y la constante de tiempo de crecimiento de las corrientes aplicadas. Rosenblueth y del Pozo (1942), dieron una relación parabólica, entre el aumento del umbral y la constante de tiempo de los pulsos aplicados. Al someter los presentes experimentos a la prueba que dichos autores indicaron, resultó que sólo algunas dieron la relación lineal establecida por ellos. Sólo podemos concluir que el músculo ventricular de la tortuga presenta acomodación a los estímulos eléctricos y que la acomodación es un proceso lento.

Otra característica de las curvas obtenidas es el hecho de no presentar una pendiente única. Esta característica fué también descrita por Rosenblueth (1942), Bernard (1942) y Sato (1950).

FIG. 4. — *Cambios en la magnitud de la acomodación, por modificaciones en la intensidad del pulso subumbral. Abscisas: porcentaje del pulso largo subumbral, con respecto a la reobase. Ordenadas: cociente* U/U_o.



Estos autores hacen mención de la existencia de un quiebre en las curvas; en las nuestras este quiebre no queda definido. Sin embargo, creemos que esta diferencia es sólo cualitativa. Varias hipótesis se han formulado para la interpretación de este accidente. Rosenblueth (1942) atribuyó la ausencia de quiebre que describieron Solandt (1936) y Screiver (1930) a las diferentes condiciones experimentales. Rosenblueth dió énfasis al hecho de haber usado nervios "in situ" y circulados. Esta explicación no podría ser aplicada a nuestras experiencias, puesto que el músculo ventricular tampoco estaba irrigado.

Por otra parte Sato y col. (1950) encontraron en el ciático de la rana un quiebre en la curva; en cambio en la fibra nerviosa aislada de este mismo tronco observaron que la relación entre la intensidad de la corriente y la constante de carga de los condensadores resultaba una línea recta. Estos autores concluyeron que la heterogeneidad en la excitabilidad del tronco nervioso estudiado, determinada por fibras nerviosas de umbral bajo y acomodación rápida y por fibras de umbral alto y acomodación lenta, explicaría este accidente en la curva.

La heterogeneidad de las fibras del músculo ventricular del corazón de tortuga, producida por la irrigación insuficiente, podría explicar, de acuerdo a esta última hipótesis, la existencia de la modificación de pendiente observada en nuestras curvas.

Los efectos de las corrientes polarizantes. — Los cambios encontrados en los umbrales son similares a los observados por Erlanger y Blair (1931-32) al iniciar o interrumpir pulsos subumbrales, aplicados al nervio. Los cambios en los que se ve un aumento del umbral inicial, corresponden a los descritos por Rosenblueth y García Ramos (1952) en las raíces lumbares del gato. La explicación de ambos efectos está en relación con las modificaciones sufridas por las respuestas locales cuando son iniciadas por estímulos de prueba catódicos de corta duración en el transcurso de la aplicación de pulsos largos catódicos y anódicos. Estas respuestas locales han sido estudiadas en el nervio, músculo estriado y en la placa neuromuscular, pero no existe un estudio de las mismas en el músculo cardíaco. Sin embargo, su existencia ha sido puesta de manifiesto por varios investigadores (Bozler, 1942-43; García Ramos y Rosenblueth, 1947). Es con estas evidencias que se explican los resultados obtenidos.

Rosenblueth y García Ramos (1952) demostraron que durante el principio de la aplicación de un pulso catódico largo a las raíces nerviosas, la magnitud de las respuestas locales bruscamente aumenta disminuyendo luego progresivamente. Al cesar el pulso, las respuestas locales primero disminuyen bruscamente de tamaño y luego aumentan progresivamente hasta alcanzar su amplitud inicial. Por lo tanto, al principio del pulso largo, el estímulo de prueba aplicado para iniciar respuesta propagada es de menor intensidad, pues se suma algebraicamente a la respuesta local producida por el paso de un pulso largo catódico. Cada vez la suma es menor y el estímulo necesario para alcanzar el umbral es mayor. Al cesar el pulso largo la respuesta local está muy disminuída de magnitud, necesitándose entonces un estímulo que está aumentado por encima del umbral para producir respuesta propagada. Con la aplicación de corrientes anódicas suceden efectos contrarios y la explicación también estaría dada por la suma de respuestas locales y estímulos eléctricos.

Con lo anterior quedarían explicados los efectos iniciales producidos por el paso de corriente directa y los efectos observados después de la aplicación de la misma. Queda sin explicar el aumento de umbrales que se observó durante la aplicación de pulsos catód-

dicos y la disminución durante las corrientes anódicas. Este cambio de umbrales es lo que se ha definido anteriormente como acomodación. De acuerdo con la nomenclatura de Rosenblueth, ésta sería positiva cuando aumentan los umbrales y negativa cuando disminuyen. El estudio detallado de las respuestas locales del músculo ventricular aclararía posiblemente el mecanismo de la acomodación.

Las respuestas repetitivas y la acomodación. — La aparición y duración de las respuestas repetitivas está estrechamente vinculada con la acomodación. Referente a este último proceso, puede hacerse la siguiente consideración. Sólo se obtienen respuestas repetitivas cuando la acomodación es lenta. La obtención de respuestas repetitivas en el músculo ventricular confirma los resultados obtenidos con los pulsos exponenciales y los pulsos largos subumbrales, de que se trata de un proceso de desarrollo temporal lento.

El hecho de que estas respuestas no aparezcan en los experimentos de ventrículo aislado, durante todo el tiempo que dura la aplicación del pulso largo, puede ser explicado por las condiciones experimentales relativamente deficientes.

Resumen

Se determinó la acomodación a estímulos eléctricos en el ventrículo aislado o perfundido del corazón de tortuga. Como estímulos se utilizaron corrientes exponencialmente crecientes. Para los cambios electrotónicos se usaron estímulos de prueba catódicos de un milisegundo de duración aplicados durante el desarrollo de pulsos largos subumbrales catódicos y anódicos. Por ambos procedimientos se encontró que el músculo cardíaco presenta el fenómeno de la acomodación y que este proceso tiene un curso temporal lento. Las características de las respuestas repetitivas confirman esta última aseveración.

Los autores agradecen a los doctores A. Rosenblueth y J. Alanís las sugestiones y consejos recibidos durante la preparación de este trabajo.

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THE VARIATION OF THE ELECTRIC RESISTANCE OF MICROELECTRODES DURING THE FLOW OF CURRENT (*)

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THE resistance of a capillary tube varies with the magnitude and direction of the electrical current passing through it. A gradual increase in the resistance is observed as the currents flow when the three following conditions are fullfilled: the current passes from within the capillary to the solution outside; the voltage applied is prolonged; the tip of the capillary is immersed in a liquid whose conductivity is less than that of the liquid within the capillary (usually Ringer's solution or cellular protoplasm). The increase of resistance of capillary tubes has imposed limitations on physiological experiments employing capillary type electrodes (microelectrodes) and through which currents greater than 10^{-9} ampers flow, in an outward direction (Weidmann, 1955; Eccles, 1957).

Taylor (1953) has suggested that this phenomenon is a consequence of the flow of the fluid of the medium into the tip of the micro-electrode, and since the conductivity of the external medium is low, the resistance of the micro-electrode rises. The flow of liquid results from a pressure difference between the two ends of the capillary tube. The data presented below confirm Taylor's (1953) suggestion.

The capillaries used were of the type described by Ling and Gerard (1949). The points were immersed in a bath containing an electrolyte solution. The solution was in contact with a chlorided silver wire with a resistance of one MΩ, which was grounded. Within the capillary, a chlorided silver wire was placed which was connected to a square pulse generator of low impedance. The other pole of the stimulator was grounded. The ends of the 1 MΩ resistance were connected to a D. C. preamplifier, or directly to a D. C. amplifier of an oscilloscope and the flow of current through the microelectrode was thus recorded.

Effect of the conductivity of the external medium on the flow of current.

Figure 1 A indicates the magnitude of the current through the micro-electrodes when pulses of 7.5 msec duration, and of 0.8, 1.2, 1.7 and 2.2. volts, were applied. Upward deflections indicate a positive voltage inside the capillaries; downward deflections, a negative voltage. The deflection corresponding to −2.2 volts was too great to be seen on the screen. These elec-

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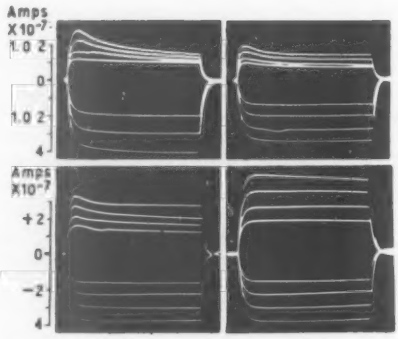

FIG. 1.—*Effect of the conductivity of the external medium on the flow of current of a micro-electrode. For explanation see text.*

trodes were immersed in a 0.17 M NaCl solution.

The same voltages were applied in figures 1 B, 1 C, and 1 D. The solutions into which the electrodes were placed were 0.5 M KCl, 2.0 M KCl, and a saturated solution of KCl, respectively. The amplification employed in these figures was half that used in figure A. The specific conductivities of the solutions in figures A, B, C and D were of increasing order. The following observations can be made from these records:

I. For a given positive voltage, the maximal current attained increases with the conductivity of the external liquid.

II. In each case, except D, for a given positive voltage, as the magnitude of the current increases, the negative slope of the descending part of the curve also increases.

III. If the conductivity of the external solution is increased, the rate of diminution of the current, after the maximal current is attained, decreases.

These observations can be interpreted from the point of view of the electro-osmotic phenomenon. If the significant pressure gradient in the micro-electrode is between the tip and the base of the cone (see equation I), when an applied voltage is positive, the flow of fluid is from the external solution into the tip of the electrode, and the mixture of the external fluid with the solution within the tip produces a gradual change in the resistance of the electrode.

A decrease in the conductivity of the liquid which enters the tip will result in a lower maximal current (Fig. I A). The liquid with the highest conductivity entering the tip of the electrode corresponds to Fig. 1 D.

Observation II is in perfect agreement with the studies of Wiedemann (1852). This author showed that: $V = A\,I$, if V be the flow of liquid expressed in ml/sec, A, a constant, and I, the current, in amperes. From this equation it follows that currents of greater magnitude will dilute the solution within the tip more rapidly, because of a greater V (Fig. I A, I B, I C), and consequently, as I increases in magnitude, so that $\frac{d\,I}{d\,t}$ increases. In addition, as the conductivity of the solution penetrating the tip is lowered, the rate at which the dilution within the tip occurs increases, and the absolute value of the above derivative, therefore, also increases.

If the direction of flow of the liquid is reversed by the application of a negative voltage, the amplitude of the current is not modified by the conductivity of the external medium.

The effect of hydrostatic pressure on the variation of resistance.

The pressure difference between two ends of a capillary tube as a consequence of electro-osmosis is given by (I) $P = 2\zeta DEL/\pi r^2$ (Glasstone, 1956) where L, is the length of the capillary tube; E, the difference of potential applied; D, the dielectric constant of

the liquid filling the pipette; ζ, the electrokinetic potential; and r, the radius of the capillary.

The pressure P is directed from outside the pipette inward, when a positive voltage is applied to it. When a hydrostatic pressure opposite in direction to P, is applied to the pipette, the flow of fluid will be modified. To obtain this effect, a micro-pipette was connected to a polyethylene tube filled with 3 M KCl. The height of the column of fluid was thus placed at different levels above the point of the electrode, which was immersed in 0.17 M NaCl. The oscillographic traces of Fig. 2 demonstrate the results obtained. These correspond to different hydrostatic pressures, with the voltage being kept constant (3.5 V). The tracing of lowest amplitude corresponds to a pressure of —5.0 cm; the next +5.0 cm; the following, +15 cm and +35 cm. This figure demonstrates clearly that with an increase of hydrostatic pressure, the maximum current tends to increase, and to remain constant.

In the graph of Fig. 2 the ordinates are the logarithm of the currents and the abscissae the logarithm of the positive voltage. The micro-pipette was subjected to a hydrostatic pressure of +20 cm. The straight line corresponding to negative voltages can be superimposed on the one in the graph. Under these conditions the microelectrode did not show any rectification.

The relation of maximum current to voltage is given by a hyperbolic equation, $I = aE^b$, with E either positive or negative. When the microelectrode was subjected to no hydrostatic pressure, the value of b was 1.23; when subjected to +20 cm, the value of b was 1.033. As the value of b approaches 1.0, the micro-electrode behaves more and more according to Ohm's law.

In some cases, when the tip of the electrode was inserted into a cell,

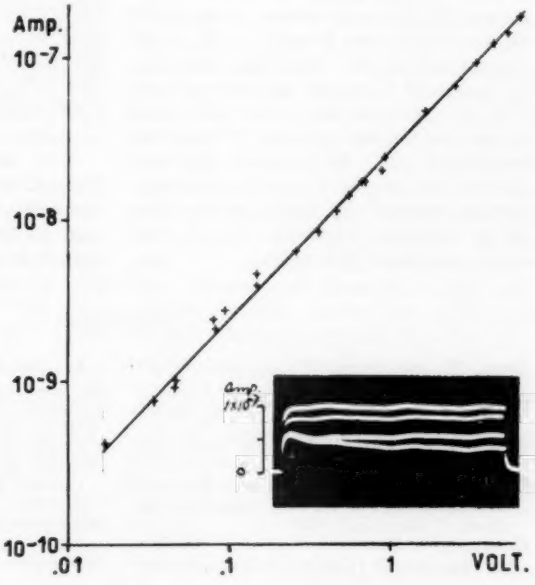

FIG. 2. — *Changes of the electrical resistance of a microelectrode caused by hydrostatic pressure. For explanation see text.*

erratic changes of the resistance occurred during the flow of current. The same phenomenon is reported by Boistel and Fatt (1958), who offered as a possible explanation the fact that certain insoluble compounds of Ca migrate into the electrode and obstruct the tip when it is made positive. These erratic changes were not affected by hydrostatic pressure.

Other authors have reported methods for correcting the increase in resistance of electrodes. Frank (1959) has shortened, under a microscope, the tip of his electrodes. According to equation (I), he has decreased the value of L, and increased the value of r, so that the value of P was diminished.

Tasaki used currents of 10^{-6} amperes, when the pH of the external volume conductor was sufficiently low. It is well known that electrokinetic potentials are greatly modified by changes in pH (Glasstone, 1956).

The dimensions of the cone of the microelectrode are also determinants of the value of the pressure needed to correct the flow of current through the electrode. As the length of the cone increases, and the diameter decreases, the pressure required becomes greater. The fact that the microelectrodes used by various authors (Eccles, 1957; Araki and Otani, 1955) had cones of different dimensions, explains the large discrepancies among the limits of the electrical currents between which they could use their electrodes.

It is evident that other methods than hydrostatic pressure can be employed to correct the gradual increase of resistance by modifying any of the factors in the electro-osmotic equation, as well as by modifying certain properties of the medium with which the electrodes are filled.

Summary

The electrical properties of capillary tubes of the type described by Ling and Gerard (1949) were studied. A gradual increase of the resistance is observed as the current flows when the three following conditions are fullfilled: the current passes from within the capillary to the solution outside; the voltage applied is prolonged; the tip of the capillary is immersed in a liquid whose conductivity is less than that of the liquid within the capillary (Fig. I). According to Taylor's (1953) suggestion these observations can be interpreted from the point of view of electro-osmotic phenomenon. In consequence, the gradual increase of resistance was modified by exerting a hydrostatic pressure on the micro-electrode (Fig. 2).

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METABOLISM OF MALTO-OLIGOSACCHARIDES (*)

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BELOFF-CHAIN et al have reported the formation of M_2 (****), M_3, M_4, etc. in tissues incubated in glucose-containing media. The synthesis of these products seemed to be closely linked with that of glycogen ([1, 2, 3]). Sie and Fishman detected the same M_{os} in rat liver and found that their changes in concentration were parallel to those of glycogen ([4, 5]). In later studies the same authors used C^{14} labeled bicarbonate as precursor and interpreted their results as evidence that glycogen was formed from M_{os} ([6]).

In a recent paper ([7]), one of us has discussed this evidence in the light of new results. It was found that a partially purified liver extract, that synthesized glycogen from UDPG ([8]), also formed M_{os} from glycogen. This preparation gave radioactive M_{os} from C^{14}-glycogen, but no label was transferred in the opposite direction, i. e. from C^{14}-M_{os} to glycogen. In the same studies it was found that the M_{os} were formed from the outer branches of glycogen, thus explaining why incubation with C^{14}-UDPG gave after 5 minutes M_{os} with much higher specific activity than that of glycogen. It was this difference in specific activity that had been used by the previous authors as main argument in favor of the roles of M_{os} as glycogen precursors.

In the present work it was found that in muscle and liver the formation of M_{os} from glycogen is catalyzed by an enzyme with the properties of an animal α-amylase. In both tissues, another enzyme was detected, which also attacks glycogen, giving rise to glucose as sole reaction product. It also libe-

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(****) Abbreviations used: M_{os}: malto-oligosaccharides; M_2: maltose; M_3: maltotriose; M_4: maltotetraose; M_5: maltopentaose; M_6: maltohexaose; UDPG: uridine diphosphate glucose.

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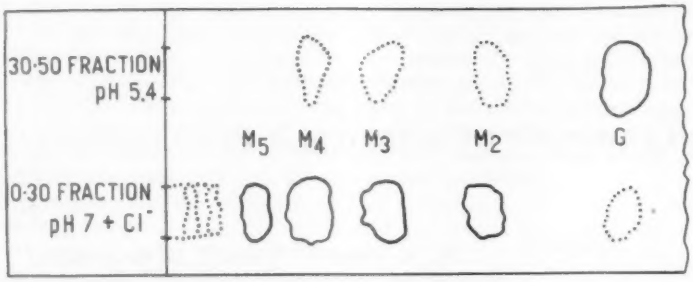


FIG. 1.—*Paper chromatography of reaction products after incubation with different muscle fractions. For 0-30 % ammonium sulfate fraction the incubation mixture contained: 16 mg of glycogen, citrate-phosphate buffer, pH 7 (sodium dibasic phosphate 22 mM, citric acid 1.6 mM), 20 mM magnesium chloride and enzyme in a total volume of 0.1 ml. Incubation time 30 minutes at 37° C. For 30-50 % ammonium sulfate fraction the same incubation mixture was used, except that the pH of the buffer was 5.4 (sodium dibasic phosphate 11 mM, citric acid 11 mM) and the magnesium chloride was omitted.*

rates glucose from M_{os} and some α-glucosides. It can thus be classified as an α 1-4 glucosidase or γ-amylase (9).

METHODS

Substrates.

Glycogen, dextran, α-methyl glucoside, and the disaccharides used were all commercial preparations. 6-bromo-2-naphthyl-α-glucopyranoside and 6-bromo-2-naphthol were obtained from California Corporation for Biochemical Research, Los Angeles, California. β-methyl glucoside was synthesized according to Koenigs and Knorr (10).

The oligosaccharides of the α 1 $\rightarrow$ 4 series (M_3, M_4, M_5 and M_6) were prepared by acid hydrolysis of amylose and fractionation on charcoal-celite columns (11). β-amylase limit dextrin was prepared from glycogen according to Hestrin (12).

Amylose and amylopectin, prepared according to Cowie and Greenwod (13), were a generous gift of Dr. Carlos E. Cardini.

A crude preparation of α-amylase inhibitor from wheat flour (14) was a gift from Dr. Clara R. Krisman.

Analytical methods.

Reducing power was determined according to Somogyi (15) and Nelson (16). Glucose was assayed in the presence of M_2 with Steinhoff procedure, as modified by Caputto et al. (17).

The method of Cohen et al. (18) for the determination of 6-bromo-2-naphthol was modified as follows: the reaction mixture was brought to 0.6 ml with water, and the pH was adjusted to 7.6 with 0.05 N NaOH, using 0.05 ml of 0.04 % phenol red as indicator. After adding 0.1 ml of tetrazotized o-dianiside (4 mg/ml freshly prepared with cold water), the mixture was well shaken and left to stand for 3 minutes. Then 0.2 ml of 80 % trichloroacetic acid and 2 ml of ethyl acetate were added, and after shaking vigorously, the mixture was centrifuged for 10 minutes at 2000 rpm. The optical density of the supernatant fluid was measured at 540 $m\mu$. The values were compared with a calibration curve obtained with 6-bromo-2-naphthol in the range between 0 and 0.03 μmoles.

Paper chromatography.

Samples were prepared for chromatography as follows: the reaction mixture was brought to 1 ml; after adding 1.25 ml of 95 % ethanol, the solution was heated to boiling, cooled and centrifuged. The supernatant fluid was passed through a 2 × 0.65 cm column of mixed bed resin (Amberlite MB-3 regenerated with acetic acid), which was then washed with 0.5 ml of water. The percolate plus the washing were evaporated to dryness over a boiling water bath under a current of air. The residues were redissolved in a small volume of water and transferred to Whatman Nº 4 paper as bands 1.5 cm wide. The chromatograms were developed with butanol-pyridine-water (6 : 4 : 3) ([19]) during 16 hours. Sugars were revealed with silver nitrate ([20]). The chromatograms were fixed by dipping in 2.5 % sodium thiosulfate and then rinsing with water.

Amylase assay.

The reaction mixture contained 0.4 mg of particulate glycogen, 5 μmoles of phosphate buffer at pH 8, and 1 μmole of NaCl, in a total volume of 0.05 ml. Incubation time was 15 minutes and the temperature 37°. The reaction was stopped by heating 2 minutes at 100° and the reducing power was determined. A pH of 8 was selected in order to avoid the action of the glucosidase.

Glucosidase assay.

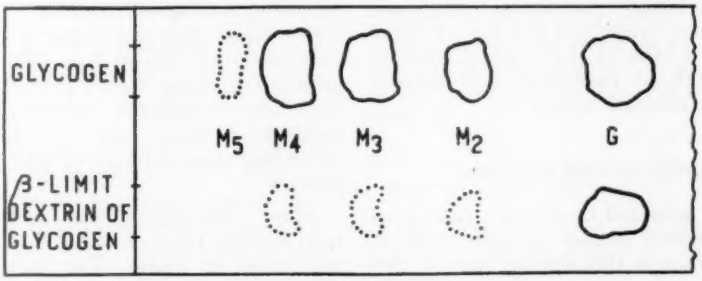
For the determination of glucosidase activity either M_2 or 6-bromo-2-naphthyl-α-glucopyranoside was used as substrate. The latter substance was dissolved in methyl-cellosolve ([21]) at a concentration of 18 mg/ml. The amount of methyl-cellosolve present in the final mixture did not inhibit when M_2 was used as substrate.

The reaction mixture contained: 0.05 ml of enzyme, 4 μmoles of M_2 or 2.6 μmoles of 6-bromo-2-naphthyl-α-glucopyranoside and 5 μmoles of acetate buffer at pH 5, in a final volume of 0.1 ml. After incubating for 30 minutes at 45°, the reaction was stopped by heating and either the reducing power, according to Caputto et al., or the liberated 6-bromo-2- naphthol was determined.

Preparation of muscle extracts.

Rats were killed by a blow in the head and bled by cutting the neck vessels. The muscles of the back and

FIG. 2. — *Paper chromatography of reaction products after incubation of glycogen and β-limit dextrin of glycogen with liver amylase. The incubation mixture contained: 2 mg of glycogen or β-limit dextrin, phosphate buffer pH 6.7, 25 mM, sodium chloride 20 mM and enzyme in a total volume of 0.2 ml. Incubation time 30 minutes at 37° C.*



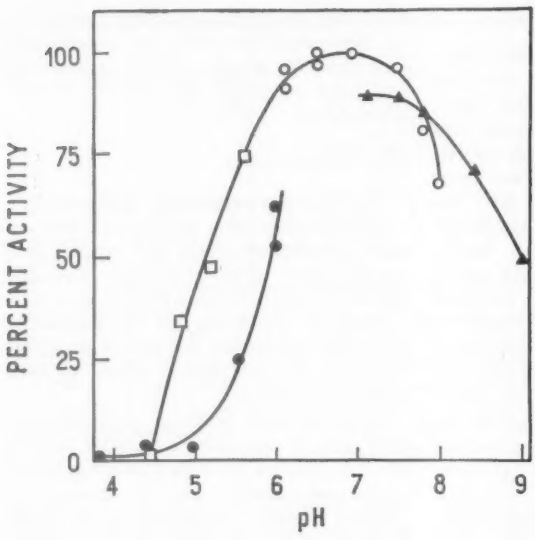


FIG. 3. — *pH optimum of the particulate fraction from liver.* ●-● *0.01 M citrate buffer;* ○-○ *0.01 M phosphate buffer;* □-□ *0.01 M acetate buffer;* ▲-▲ *0.01 M tris (hidroxymethyl)-aminomethane-Cl-buffer.*

hind legs were removed and homogenized in a blendor with thrice their volume of distilled water during 5 minutes. The homogenate was filtered through muslin and brought rapidly to 50°. This temperature was maintained during 10 minutes and then the extract was cooled rapidly. In this step the enzymes under study did not lose activity, while a large quantity of inactive protein was denatured. The precipitated protein was centrifuged off during 10 minutes at 15,000 *g*, and the supernatant fluid was brought to 50 % saturation with solid ammonium sulfate. After centrifuging, the precipitate was redissolved in a small volume (about 1/50th of the original volume) and dialyzed overnight with stirring against distilled water.

Preparation of liver fractions.

Rats were fed by stomach tube 2 ml of saturated sucrose solution three or four times a day during one or two days in addition to the normal *ad-libitum diet*. The animals were killed as described above and the livers were homogenized with three volumes of distilled water in a Potter type glass homogenizer. Whole cells, cell débris and nuclei were centrifuged off during 10 minutes at 5,000 *g*. The supernatant fluid was then spun at 25,000 *g* during 10 minutes. The translucent pellet that adhered firmly to the bottom of the tube will be referred to as "25,000 *g* particulate glycogen". The supernatant fluid was again centrifuged, in a Spinco model L preparative ultracentrifuge at 105,000 *g* during 30 minutes.

The new glycogen pellet is called "105,000 *g* particulate glycogen". Both glycogen fractions were equally active. They were suspended, individually or together, in water and reprecipitated by adding three volumes of cold (—15°) acetone.

After centrifuging, the precipitate was washed twice with cold acetone and dried *in vacuo*. The particulate

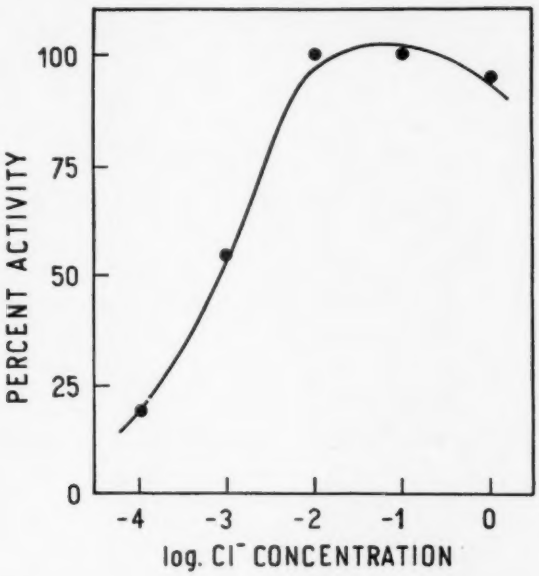


FIG. 4.—*Effect of chloride on the activity of the particulate fraction from liver.*

glycogen, when dried in this way, maintains its activity during several months at room temperature.

The supernatant fluid from the 105,000 *g* centrifugation was used as glucosidase preparation.

RESULTS

Muscle extracts.

During the first stages of purification (heating and first ammonium sulfate precipitation) the formation of M_{os} was estimated by measuring the liberation of reducing power from glycogen. When the results were checked by paper chromatography, it was found however that in addition to M_{os}, glucose also was formed in substantial amounts, and the ratio M_{os} to glucose varied according to the conditions. For instance, after incubation at pH 7 to 8 in the presence of 0.01 M Cl^-, the amount of M_{os} was far greater than that of glucose, while at pH 5 the inverse result was obtained. This indicated that two different enzymatic activities might be involved in the process and an attempt was then made to separate them. From then on, the different fractions were incubated under the two above mentioned conditions, and the reaction products were identified by paper chromatography. Although a complete separation could not be obtained, the best results were given by ammonium sulfate fractionation. The fraction that precipitated between 0 and 0.30 saturation was more active in the formation of M_{os}, while that brought down between 0.30 and 0.50 saturation gave rise preferentially to glucose, as can be seen in figure 1.

Action of the two fractions on different substrates.

The results obtained by submitting to paper chromatography the reaction products formed by each of the two

TABLE I

Action of muscle fractions on different substrates

Incubation mixtures were the same as those used in figure 1. Substrate final concentration was 10-15 mg/ml for polysaccharides and 2-5 μmole/ml for other substrates

Substrates	Ammonium Sulfate Fractions					
	0-30 Fraction			30-50 Fraction		
	Hours of incubation	Product	Amount formed	Hours of incubation	Product	Amount formed
Glycogen	0.5	M_{os}	+++	0.5	Glucose	++
Amylose	1	M_{os}	++	1	—	—
				16	Glucose	+
Dextran	16	—	—	16	Glucose	+
β-limit dextrin of glycogen	0.5	M_{os}	+			
M_2				0.5	Glucose	++
M_3	1	M_2, Glucose	±	0.5	Glucose	++
M_4	1	M_2	++	0.5	Glucose	++
M_5	1	M_3, M_2	++			
M_6	1	M_4, M_3, M_2	++			
Mixture of M_{os}				16	Glucose	++++
α-methyl glucoside	16	—	—	16	Glucose	+
β-methyl glucoside	16	—	—	16	—	—
Sucrose				16	—	—
Cellobiose				16	—	—
Isomaltose				16	—	—

fractions are summarized in Table I. These results show qualitatively the action of each enzyme on different substrates. The fraction from 0 to 0.3 saturation gives M_{os} with glycogen and amylose. It also acts on β-limit dextrin from glycogen, but at a slower rate, and with a decrease in formation of the higher M_{os}, as shown in figure 2. It can also be seen in Table I that this fraction cleaves the M_{os} to smaller fragments. The action on M_3 is very slow and may be due to contaminant enzymes. On the other hand dextran and methyl glucoside are not attacked.

The other fraction (between 0.30 and 0.50 saturation) liberates glucose from glycogen as well as from M_{os}, including M_2. Its action on amylose is very slow, and appreciable increase in reducing power can be measured only after 16 hours of incubation. Some glucose seems also to be liberated from dextran in 16 hours but this result requires confirmation with more purified preparations. Glucose is split off from α-methyl glucoside, but not from the β-anomer, or from sucrose, cellobiose (β-glucosyl 1-4 glucose) or isomaltose (α-glucosyl 1-6 glucose).

Liver extracts.

Liver extracts also showed the presence of two enzymatic activities, similar to those described above for muscle. It was not possible to separate the two enzymes in extracts from starved or normally fed rats. When the animals were submitted to previous forced feeding with sucrose, the M_{os} forming activity was found to be associated with the particulate glycogen ([22]), while the glucose-liberating activity was recovered in the 105,000 *g* supernate. Since in these fractions the two activities were much better separated than in those of muscle, a quantitative study was attempted with the liver enzymes.

Properties of the particulate fraction.

pH optimum. — As shown in figure 3, the pH optimum was found at about pH 6.8, a value that falls in the range (between 6 and 7) reported for several mammalian amylases ([9]).

Chloride requirement. — The enzymatic activity of this fraction is strongly activated by chloride ions, with an optimum concentration between 10^{-1} and 10^{-2} M as it can be seen in figure 4. This activation is also similar to that found for other α-amylases ([9]).

Inhibitors. — In Table II the inhibitory actions of Zn^{++} on liver and saliva amylase are compared. In both cases ethylenediamine tetraacetic acid relieves the inhibition. It can also be seen that both activities are diminished by the wheat inhibitor described by Kneen et al. ([14, 23]). In figure 3 it is shown that the activity is lower with citrate than with acetate or phosphate buffer. The inhibiting action of citrate on some α-amylases has been described by Street ([24]).

Properties of the 105,000 g supernate.

Kinetics. — Figure 5 shows the effect of substrate concentration on the rate of hydrolysis. Half the maximum velocity is obtained with 5×10^{-3} M M_2.

pH optimum. — The same pH optimum, between 4 and 5, was obtained with either M_2 of 6-bromo-2-naphthyl-α-glucopyranoside as substrate, as it can be seen in figure 6.

Discussion

One problem that remained unsolved in a previous paper ([7]) was the nature of the enzymes that catalyze the formation of M_{os} from glycogen. The present findings indicate that, in muscle as well as in liver, glycogen can be

TABLE II

Action of inhibitors

Saliva was centrifuged 10 minutes at 25 000 *g* and the supernate was used after dilution with water

Additions (Final Concentration)	Percent Activity	
	Saliva	Particulate fraction
Zn 2×10^{-3} M	1	0
Zn 2×10^{-3} M EDTA* 4×10^{-4}	3	0
Zn 2×10^{-4} M EDTA 4×10^{-4}	82	65
Wheat inhibitor 1.35 mg/ml	38	36
Wheat inhibitor 2.7 mg/ml	9	19
Wheat inhibitor 5.4 mg/ml	4	13

(*) EDTA = ethylenediamine tetraacetic acid.

hydrolyzed in two ways: one, through the breaking up of chains into fragments of several glucose units —the M_{os}— the other by liberating one glucose at a time. The enzyme that catalyzes the first type of hydrolysis has the properties of an α-amylase. The pH optimum, the Cl^- requirement, the action of inhibitors, and the activity towards different substrates, are all similar to those described for this group of enzymes. The reaction is faster with glycogen than with the M_{os}, and is slowest with M_3, so much so that it is doubtful whether the cleavage of this substrate is due to the α-amylase or to some contaminating enzyme. Whether M_3 is hydrolized or not by α-amylases is a much debated problem ([25, 26, 27, 28]), but recent evidence ([29, 30, 31]) is strongly in favor of an affirmative answer.

The experiment shown in figure 2 confirms the previous hypothesis ([7]), that the M_{os} are formed mainly from the external branches of glycogen. In the hydrolysis of β-amylase limit dextrins, whose exterior branches only have 2 or 3 glucose units, most of the reaction products must come from the inner chains. These are relatively short (3 to 4 glucose residues in average) and, in addition, every branch point (1-6 linkage) protects some of the neighboring α 1-4 linkages from enzyme action ([32]). It can thus be easily understood why the main linear saccharide obtained with this substrate is M_2. In addition, branched oligosaccharides are formed, that move only slightly from the starting line of the chromatograms.

This provides an explanation for the fact that, with C^{14}-UDPG precursor, the specific activity of the M_{os} formed, decreased progressively from M_5 to M_2 ([7]). Thus, when, as in this case, one starts from a glycogen pre-

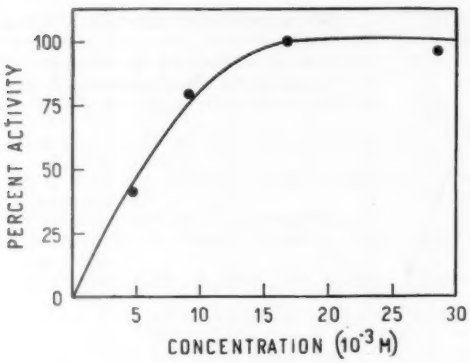


FIG. 5. — *Effect of maltose concentration on the rate of hydrolysis with liver glucosidase.*

valently labeled in the external branches, M_2 and the lower M_{os}, that originate partly from the inner, less radioactive chains, will have a lower specific activity than the higher M_{os}, that originate mainly from the outer, more radioactive branches.

The presence of α-amylase in liver and other organs has been the subject of a controversy. After the discovery of the phosphoroclastic pathway in the degradation of glycogen, the amylolytic pathway was discarded [33, 34] and the amylase in liver was supposed to result from contamination with blood plasma [35]. McGeachin has studied this problem exhaustively [36] and has reported evidence that liver α-amylase is formed in the hepatic cell and is independent from the circulating amylase [37, 38, 39]. Other authors have found that under certain conditions liver contains between 5 and 10 times more amylase that can be ascribed to the blood contained in the tissue [40].

The role of this amylase in the metabolism of carbohydrates is uncertain. Dreiling et al. [41] and Levin [42] have supposed that it might participate somehow in the process of glycogenolysis. On the other hand Smith [43] and McGeachin and Potter [38] have advanced the hypothesis that it might take a part in glycogen synthesis.

The second enzyme described in this paper hydrolyzes glycogen, α 1-4 oligosaccharides and M_2 to glucose. Therefore, it belongs to the group of the variously called γ-amylases, glucoamylases [9], or 1-4 glucosidases [44]. Other examples are the glucoamylase [45, 46] and α 1-4 amyloglucosidase [47] found in moulds and the maltase from horse serum described by Lieberman and Eto [48]. The latter enzyme differs from the liver enzyme, since its pH optimum is higher (6.3-7.2), it does not split α-methyl glucoside and its Km is ten times smaller. The properties of the liver enzyme are more akin to those of the mould glucosidases. No transglucosidase activity could so far be demonstrated with the liver enzyme. When it was incubated with glycogen, in the presence of C^{14}-glucose, no labeled M_2 was found to be formed.

The low pH optimum of this enzyme suggests that its activity is probably very low under physiological conditions. Nevertheless, the amylase and α-glucosidase could act in concert to liberate glucose from glycogen, according to the equation:

$$\text{Glycogen} \xrightarrow{\alpha\text{-amylase}} M_{os} \xrightarrow[\text{sidase}]{\alpha\text{ 1-4 gluco-}} \text{glucose}$$

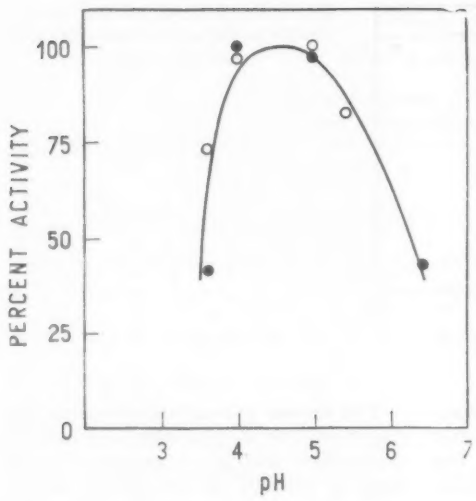


FIG. 6. — *pH optimum of the 105 000 g supernate from liver.* ○-○ *with maltose as substrate;* ●-● *with 6-bromo-2-naphtyl-α-glucopyranoside as substrate.*

It is now generally accepted that the transformation of glycogen into glucose takes place through the action of several enzymes: phosphorylase, phosphoglucomutase and glucose-6-phosphatase ([33, 49]) for α 1-4 linked residues and amylo 1,6 glucosidase ([50]) for α 1-6 linked residues. When glucose-6-phosphatase is absent ([51]) (like in muscle), glucose would only be formed by amylo 1,6 glucosidase.

Whether both the phosphoroclastic and the amylolytic pathways are operative in animal tissues must await further investigations.

SUMMARY

Muscle and liver extracts were found to contain two enzymes acting on glycogen. The two activities could be separated partially in muscle extracts and more completely in liver extracts. In the latter preparations one of the enzymes was found to sediment together with the particulate glycogen fraction, while the other remained in the supernate. The particulate enzyme showed the properties or an α-amylase, while the soluble enzyme liberated glucose from both glycogen and maltooligosaccharides and is therefore considered a γ-amylase. The α-amylase is the enzyme responsible for the previously reported formation of oligosaccharides from glycogen.

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PROCEEDINGS OF THE SOCIETY OF BIOLOGY
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Studies on the regeneration of legs in "Triatoma infestans" (Klug, 1834) (Hemiptera, Reduviidae). Fernando M. A. Corrêa.

The regenerative capacity of legs in *Triatoma infestans* (Klug, 1934) is described for the first time. It is similar to that observed by Lüscher (1948) in *Rhodnius prolixus* Stal, 1859, that is, it appears at the femoro-tibial joint and increases gradually in a distal direction. It was also observed that the size of the regenerated leg depends on the period of time which elapses between the amputation and the next moult, the size been larger when the leg is amputated earlier in a given stage.

Since it has been demonstrated for other insects that the final size of a regenerated structure usually depends on the period of time elapsed between amputation and the last moult (Bodenstein, 1953), the author tried to modify the duration of nymphal stages by varying the frequency of blood meals. Altrough the number of insects used was small, there is an indication that the larger the duration of the life cycle the larger the final size of regenerated legs in *T. infestans*.

Pulmonary lesions produced by anti-placenta serum in rats. Helio Lourenço de Oliveira and José Augusto Laus Filho. *(Department of Clinical Medicine, Faculty of Medicine, Ribeirão Prêto, São Paulo, Brazil).*

Experimental pulmonary lesions induced in rats by intravenous injection of nephrotoxic sera (rabbit anti-rat-kidney and anti-rat-lung sera) were described by Laus et al. (1957) and further studied by Laus (1958). Immuno-sera devoid of appreciable nephrotoxicity (anti-rat-intestine, anti-rat-spleen and anti-rat-serum) did not produce the pulmonary changes even when given in dosis large enough to produce extensive hemorrhagic lesions in the intestinal tract.

The view that the pneumotoxicity of the anti-sera is related to their nephrotoxicity is supported by the results of the present experiments with rabbit anti-rat-placenta serum. This, as well as anti-kidney and anti-lung sera, is known to convey nephrotoxic factors (Seegal and Loeb, 1946).

Intravenous injection of the anti-placenta serum produced the same acute functional manifestations (dyspnoea and cyanosis, with death of some animals with acute pulmonary edema), the same macroscopic changes in the lungs (edema and hemorrhage) and the same histologic picture as seen in rats receiving either anti-lung or anti-kidney serum.

Anti-placenta serum previously absorbed with rat intestine, liver or spleen still retained its pneumotoxicity. When absorbed with kidney tissue it lost that activity. Such results are shown in the table where lung changes are classified macroscopically as follows: 0 = no significant deviation from normal; + = edema and hemorrhage; ++ = severe edema and hemorrhage, with trachea filled with frothy secretion (and early death in the acute stage). The distribution, according to the degrees of pulmonary lesions, of 40 rats that received different treatment is:

Degrees of pulmonary lesions ..	0	+	++
Non-absorbed serum	2	5	10
Serum absorbed with liver	2	3	3
Serum absorbed with intestine .	0	2	0
Serum absorbed with spleen ...	2	2	0
Serum absorbed with kidney ..	9	0	0

The results may be explained by the existence in the three organs: placenta, kidney and lung, of at least one commom antigen that is responsible for the pneumotoxicity of the corresponding anti-sera. Baxter and Goodman (1956) reached this conclusion in relation to the nephrotoxicity of the same anti-sera. It is probable that the pulmonary and the renal lesions are the results of the reaction of the same antibody with the commom antigen in both organs.

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June 17, 1960

Effects of cholinergic drugs on the esophagus of patients with Chagas' disease.

RENATO ALVES DE GODOY AND CLOVIS BÜHLER VIEIRA. *(Department of Medicine, Faculty of Medicine, Ribeirão Prêto, São Paulo, Brazil).*

Recording by a kymographic method the variations of air pressure inside a small balloon located in the lower esophagus, the authors have studied the motility of this organ after administration of pilocarpine hydrochloride and acetylbetamethylcholine chloride to patients with Chagas' disease. This infectious disease, caused by the protozoa Schizotrypanum Cruzi is practically responsible for all cases of megaesophagus in Brazil.

Four healthy students and fourteen patients with Chagas' disease were studied with the aid of this technique. Five patients had megaesophagus and three had normal esophagus. The other six were patients without dysphagia and with a normal sized organ, but they had an emptying time, measured by a special radiographic technique, considered lengthened.

The maximal doses of the mentioned drugs employed in this study were 0.1 mg/Kg body weight.

The controls after an intramuscular injection of a dose from 0.05 to 0.1 mg/Kg weight of methacholine or pilocarpine did not show any alteration or at most a slight increase of esophagus motility and muscular tonus. The five patients with megaesophagus, after the injection of the same dose of the drugs, showed a considerable increase of organ motility and tonus. Of the six patients with delayed emptying time, three reacted like the megaesophagus patients and the other reacted as the normal controls. The three patients with the disease and without esophagus disfunction also reacted as normals.

Most of the research in this subject is done in patients with megaesophagus, where considerable alteration of motility and tonus is found. This study indicates that even esophagus with incipient disfunction may be hyperreactive to parasympathomimetic agents.

November 14, 1960

Studies on histamine liberation in the presence of metabolic inhibitors.

A. M. ROTHSCHILD AND ITAMAR VUGMAN. *(Department of Pharmacology, Faculty of Medicine, Ribeirão Prêto, São Paulo, Brazil).*

Histamine liberation *in vitro* was studied in the isolated rat diaphragm using compound 48/80, a condensation product of formaldehyde and p-methoxyphenylmethylamine, which acts as a powerful releaser of rat tissue histamine.

This process is inhibited by uncoupling agents like 2,4 dinitrophenol (DNP), sodium salicylate, sodium oleate and thiopental, as well as by cyanide or oxygen lack. It was found that the action of these inhibitors was only exerted when glucose was absent from the Krebs-Ringer phosphate buffer used as incubation medium. The addition of glucose produced complete reversal of the inhibitory effect of DNP, sodium salicylate, oleate and thiopental and 80 % reversion of the effect of sodium cyanide and anoxia.

Krebs cycle intermediates like succinate or fumarate were unable to affect the inhibitory action of DNP or NaCN. The inhibitory action of a SH-group blocking agent like p-chloro mercuri-benzoate as well as that of sodium fluoride was not affected by the presence of glucose. The effect of sodium fluoride could however be partly overcome by the addition of calcium ions, and was totally reversed by a mixture of calcium and magnesium ions.

The effectiveness of glucose in abolishing the inhibitory action of DNP and NaCN was also apparent upon microscopic examination of mast cells in spreads of rat mesentery incubated with compound 48/80. Granule extrusion, the typical results of the action of compound 48/80 on these cells, was fully prevented by the presence of DNP or NaCN, but was extensive when glucose was present in the medium as well.

Studies on the oxygen uptake of the isolated rat diaphragm in the Warburg apparatus, revealed that at concentrations which inhibited histamine release, DNP initially enhanced, and subsequently depressed oxygen consumption by the tissues. The presence of glucose did not influence this effect of DNP. In contrast, the presence of succinate allowed for normal respiratory activity to be maintained for a much longer period of time, during which however, the inhibition of histamine release was not reversed.

The inhibition of histamine release by uncouplers of oxidative phosphorylation, by cyanide and by anoxia indicates that the synthesis or use of energy-rich compounds are essential steps for the release of histamine by compound 48/80.

It is known that the anaerobic synthesis of ATP through the glycolytic pathway, is insensitive to the inhibitory effects of DNP and other uncoupling agents. The effectiveness of glucose in promoting this release under conditions in which oxidative metabolism is severely repressed, suggests that the mast cell uses anaerobic glycolysis, or possibly some other available phase of glucose metabolism, as an additional reserve mechanism to ensure histamine release under conditions in which oxidative "high energy bond" synthesis is suppressed.

Effect of glucose and insulin on the histamine release by compound 48/80 from alloxan diabetic rats. ITAMAR VUGMAN AND ADOLFO M. ROTHSCHILD. *(Department of Pharmacology, Faculty of Medicine, Ribeirão Prêto, São Paulo, Brazil).*

Release of histamine by compound 48/80 from the isolated diaphragm of alloxan diabetic rats was studied. When compared to that of normal rats it was 25 % lower in the diabetic animal. Though glucose increased histamine release from the tissue of diabetic rats, this release only reached the normal control levels when glucose plus insulin were added to the incubation medium.

Metabolic inhibitors such as 2,4-dinitrophenol (DNP) and sodium cyanide (NaCN) prevented the release of histamine. In the presence of glucose, inhibition by DNP was reduced by 82 % and inhibition due to NaCN, by 61 %. When glucose plus insulin were added, the inhibitory action of DNP was totally abolished. In contrast, inhibition due to NaCN was only partially reversed remaining at a level of 27 % inhibition, when glucose and insulin were added.

The results show that compound 48/80 is able to release histamine from the diaphragm of alloxan diabetic rats. In comparison with normal tissues this release is partially impaired. Insulin plus glucose were able to restore the level of histamine release to that obtained with normal rats. The observation that glucose was able to partially revert the inhibition caused by DNP and NaCN, indicates that mast cells of alloxan diabetic rats were able to utilize part of the glucose added to the incubation medium. Insulin was able to reverse these inhibitions still further; this indicates that when used in conjunction with glucose, the hormone is able to promote the action of compound 48/80 even in tissues treated with DNP or NaCN.

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centímetro	cm	cm	centímetro cúbico	cm^3	cc
milímetro	mm	mm	mililitro	ml	ml
micrón	μ	μ	kilogramo	kg	kg
milimicrón	$m\mu$	$m\mu$	gramo	g	gm
Angström	Å	Å	miligramo	mg	mg
microgramo	μg	μg	miliequivalente	mEq	mEq
hora	h	hr	Curie	c	c
minuto	m	Min	Milicurie	mc	mc
segundo	s	sec	Microcurie	μC	μC
milisegundo	ms	msec	por ciento	%	%

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